

SYNCHRONIZATION OF OSCILLATORS AND ITS MOLECULAR  
MECHANISM(S) IN CYANOBACTERIA

By

Jing Xiong

Thesis

Submitted to the Faculty of the  
Graduate School of Vanderbilt University  
in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

in

Biological Sciences

May, 2014

Nashville, Tennessee

Approved:

Dr. Carl Johnson

Dr. Katherine Friedman

To my amazing parents

And

To my beloved husband, Chao Liu, infinitely supportive

## ACKNOWLEDGEMENTS

This work would not have been possible without the financial support of the National Institute of General Medical Sciences, to Dr. Carl Johnson, the Vanderbilt International Scholar Program, and Marine Biological Laboratory. I am especially indebted to my mentor Dr. Carl Johnson, in the Department of Sciences, who has been always supportive of my career goals and inspiring my exploration in biology. His hard-working attitude and passion of science have shown me, by his example, what a great scientist should be.

I would specifically thank Dr. Terry Page, Brandt Eichman, James Patton and David Piston for their great advice and discussions during this project and Dr. Mark Byrne for the valuable suggestions on the computational simulation project.

I am grateful to all of those with whom I have had the pleasure to work during all these years. Dr. Ximing Qin and Dr. Tetsuya Mori offered me guidance and advice. I also appreciate the help from other colleagues in the Johnson lab, Dr. Yao Xu, Dr. Yunfei Zhang, Dr. shuqun Xu, Peijun Ma, Kathryn Campbell and Ashely Libby.

Nobody has been more important to me than the members of my family. I would like to thank my parents, whose love is always with me wherever I am. Most importantly, I wish to thank my loving and supportive husband, Chao, and our wonderful dog, Mikey, who provide infinite happiness to our family.

## TABLE OF CONTENTS

	Page
DEDICATION.....	ii
ACKNOWLEDGEMENTS .....	iii
LIST OF TABLES.....	vi
LIST OF FIGURES.....	vii
Chapter	
I. INTRODUCTION: CYANOBACTERIA AS A MODEL SYSTEM TO STUDY	
CIRCADIAN CLOCK .....	1
Circadian rhythms and its importance.....	1
Three criteria in the determination of circadian clock.....	2
Cyanobacteria as a model system to study the circadian clock.....	3
Probe the circadian rhythms using luciferase reporters.....	4
Core clock genes in cyanobacteria.....	6
KaiABC system in cyanobacteria.....	6
Transcriptional and translational feedback loop (TTFL) model.....	8
A post-translational oscillator (PTO) found in cyanobacteria .....	8
II. SYNCHRONIZATION OF OSCILLATORS WITH DIFFERENT PERIODS <i>IN VITRO</i>	
AND <i>IN VIVO</i> .....	11
Introduction.....	11
Results.....	15

Synchronization of oscillators with different periods <i>in vivo</i> .....	15
Period changes according to different expression levels of KaiC mutants .....	17
Synchronization of oscillators with different periods <i>in vitro</i> .....	24
Computational simulation of synchronization of oscillators with different period ....	32
Discussion .....	42
Materials and Methods .....	46
Strains, Culture Conditions, and Luminescence Assay of <i>In Vivo</i> Rhythms.....	46
Transformation of cyanobacteria.....	47
Preparation of Kai proteins.....	48
<i>In vitro</i> reactions.....	49
Analysis of protein interactions among three Kai proteins by Native-PAGE.....	49
Model description .....	50
REFERENCES.....	53

## LIST OF TABLES

Table	Page
1. Phenotypes, nucleotide mutations, and amino acid mutations of different KaiC mutants used in the study.....	49
2. Model parameters and rates. ....	55

## LIST OF FIGURES

Figure	Page
1. Circadian rhythms of bioluminescence of <i>S.elongatus</i> PCC 7942 AMC149 strain in continuous light conditions.....	5
2. <i>In vitro</i> oscillation of KaiC phosphorylation states. ....	10
3. Synchronization of KaiC phosphorylation rhythms with different phases.....	12
4. A generalized diagram for the strains used in the study.....	19
5. Synchronization of oscillators with different periods <i>in vivo</i> .....	21
6. Quantification of the synchronization experiments (WT, T42S and T415A) <i>in vivo</i> . ....	22
7. Quantification of the synchronization experiments (WT, T42S and F470Y) <i>in vivo</i> . ....	23
8. 3Myc tagged KaiC retained a strong <i>in vitro</i> phosphorylation rhythm similar to WT KaiC.....	27
9. KaiC <sup>T42S</sup> mutant possessed a strong <i>in vitro</i> phosphorylation rhythm with a long period. ....	28
10. Two oscillators (3myc-KaiC and KaiC <sup>T42S</sup> ) with different periods synchronized with a shared period and phase. ....	29
11. KaiC <sup>P248A</sup> mutant possessed a strong <i>in vitro</i> phosphorylation rhythm with a short period. ....	30
12. Two oscillators (3myc-KaiC and KaiC <sup>P248A</sup> ) with different periods	

synchronized with a shared period and phase, but showed dampened rhythms.....	31
13. Model prediction of synchronization of oscillators with different periods due to KaiA-KaiC phosphorylation rate decrease as a function of time in the <i>in vitro</i> oscillation reaction. ....	35
14. Model prediction of synchronization of oscillators with different periods due to KaiA-KaiC phosphorylation rate increase as a function of time in the <i>in vitro</i> oscillation reaction. ....	36
15. Model prediction of synchronization of oscillators with different periods due to KaiC* dephosphorylation rate decrease as a function of time in the <i>in vitro</i> oscillation reaction. ....	37
16. Model prediction of synchronization of oscillators with different periods due to KaiC* dephosphorylation rate increase as a function of time in the <i>in vitro</i> oscillation reaction. ....	38
17. Model prediction of synchronization of oscillators with different periods due to KaiA-KaiC on/off rate decrease as a function of time in the <i>in vitro</i> oscillation reaction.....	39
18. Model prediction of synchronization of oscillators with different periods due to KaiA-KaiC on/off rate increase as a function of time in the <i>in vitro</i> oscillation reaction.....	40
19. Model prediction of synchronization of oscillators with different phases as a function of time in the <i>in vitro</i> oscillation reaction in the presence or absence of monomer exchange. ....	41



## CHAPTER I

### INTRODUCTION: CYANOBACTERIA AS A MODEL SYSTEM TO STUDY CIRCADIAN CLOCK

#### *Circadian rhythms and their importance*

The circadian clock has been found universally in eukaryotes and is also observed in prokaryotic cyanobacteria. It is an intrinsic mechanism that allows organisms to rhythmically regulate gene expression and thereby mediate a great number of biological processes<sup>1,2</sup>. For instance, the endogenous clock in humans plays a major role in the sleep/wake cycle, body temperature cycle and even endocrine cycles with a period approximate to 24 hours in duration<sup>2</sup>. Various human disorders result from disturbance to the circadian rhythm, including seasonal affective disorder (SAD), delayed sleep phase syndrome (DSPS), and advanced sleep phase syndrome (ASPS)<sup>3</sup>. Additionally, a number of problems such as obesity and diabetes were originally considered to be associated with lifestyle and genetic factors; however, recent research has indicated that disruption of circadian timing system may also contribute to the development of these metabolic disorders. Studies carried out on mice have demonstrated that mutations or deletions of the *clock* gene lead to several metabolic disorders, including hyperphagia, obesity and altered glucose metabolism<sup>4</sup>. Moreover, *Bmal1*<sup>-/-</sup> mice have reduced lifespan and suffer from symptoms of premature aging and age-related pathologies<sup>5</sup>.

### *Three criteria in the determination of circadian clock*

Not all biological rhythms with a period of 24 hours are considered circadian rhythms. Circadian rhythms have three diagnostic characteristics<sup>2</sup>. First of all, under constant conditions (i.e. constant light or darkness) circadian rhythms display an endogenous free running period that persists with a period close to 24 hours. This criterion can be used to distinguish circadian rhythms from biological rhythms that merely respond to daily external cues, such as light and temperature cycles. Furthermore, circadian rhythms are entrainable by external stimuli. Entrainment means that an internal circadian clock conforms to the period of the environment with a stable phase relationship, when their periods are not exactly the same. In other words, following the exposure to a different environmental cycle (changed phase or period), circadian rhythm can be gradually reset and adapted to the new scheme. An entrained rhythm by the environment stimuli has to show that (1) Their periods have to be the same with a stable, unique phase angle, and (2) that after releasing the rhythm from the environment stimuli, its free-running period resumes with a phase angle determined by the previous stimuli cycle. Lastly, the period lengths of the free-running rhythm are comparable over a range of physiological temperatures ( $Q_{10} \sim 1$ ). Organisms including humans are exposed to fluctuations of temperature due to seasonal and weather changes. However, our circadian clock has maintains an ~24-hour period spanning different temperature conditions, and this is known as temperature compensation<sup>2</sup>.

### *Cyanobacteria as a model system to study the circadian clock*

Early on, it was believed that circadian rhythms only exist in eukaryotes, since an endogenous timekeeper with a ~24 hour period did not seem to be useful for prokaryotes that often divide multiple times within a day. However, many strains of chlorophyll-containing prokaryotic bacteria (cyanobacteria) are capable of autotrophic photosynthesis as well as nitrogen fixation. This observation was quite a puzzle since the process of nitrogen fixation is severely inhibited by the presence of oxygen, which is released mostly as a by-product of photosynthesis as reported in 1985. It turned out that the rhythm of nitrogenase activity peaks in the subjective night in the cyanobacterium *Oscillatoria* sp. even under constant light condition (LL)<sup>6</sup>. A year later, another group reported that rhythmic nitrogen fixation activity was temporally separated from photosynthesis as a way to reconcile these two contradictory processes, since photosynthesis peaked during the subjective daytime while nitrogen fixation was active during subjective night. This finding was based on studies in the unicellular marine cyanobacteria *Synechococcus* spp. Miami BG43511 and 43522<sup>7</sup>. Meanwhile, Huang and colleagues further demonstrated that another species of cyanobacteria clearly exhibit circadian rhythms of nitrogen fixation activity, which confirmed that circadian rhythms with a ~24 hour period might be found even in prokaryotes<sup>8</sup>. Due to the easily manipulatable genetics in cyanobacteria, they have been used as a model system to study the circadian clock ever since<sup>9</sup>.

### *Probing circadian rhythms using luciferase reporters*

The unicellular freshwater species *S.elongatus* PCC7942 was considered as a terrific candidate to work with since they have a relatively small genome and can also be genetically manipulated conveniently – they can be transformed with exogenous DNA through homologous recombination with relatively high efficiency<sup>10</sup>. Pioneering investigations of introducing reporter genes into cyanobacteria were performed in the early 1990s, when this strain was transformed with a construct containing the *Vibrio harveyi* luciferase gene set *luxAB* fused to the promoter *psbA1* (a *Synechococcus* photosystem II gene) into the neutral site I on the chromosome<sup>11</sup>. This reporter strain was named AMC149. Utilizing this reporter strain, a eukaryotic-like circadian rhythm was revealed in cyanobacteria. The emitted bioluminescence matched all three criteria mentioned above [figure1]. In particular, the rhythm persists in constant light conditions (LL) after one LD cycle entrainment with a period of ~24 hours (endogenous). Secondly, after being entrained previously to LD cycles that were 12 hr out of phase, the time course of bioluminescence in LL showed robust opposite-phase-traces (entrainable). Finally, the bioluminescence displayed similar free running periods under three different constant temperatures: 25, 30 and 36°C, leading to a calculated Q<sub>10</sub> of 1.1 (temperature compensated).

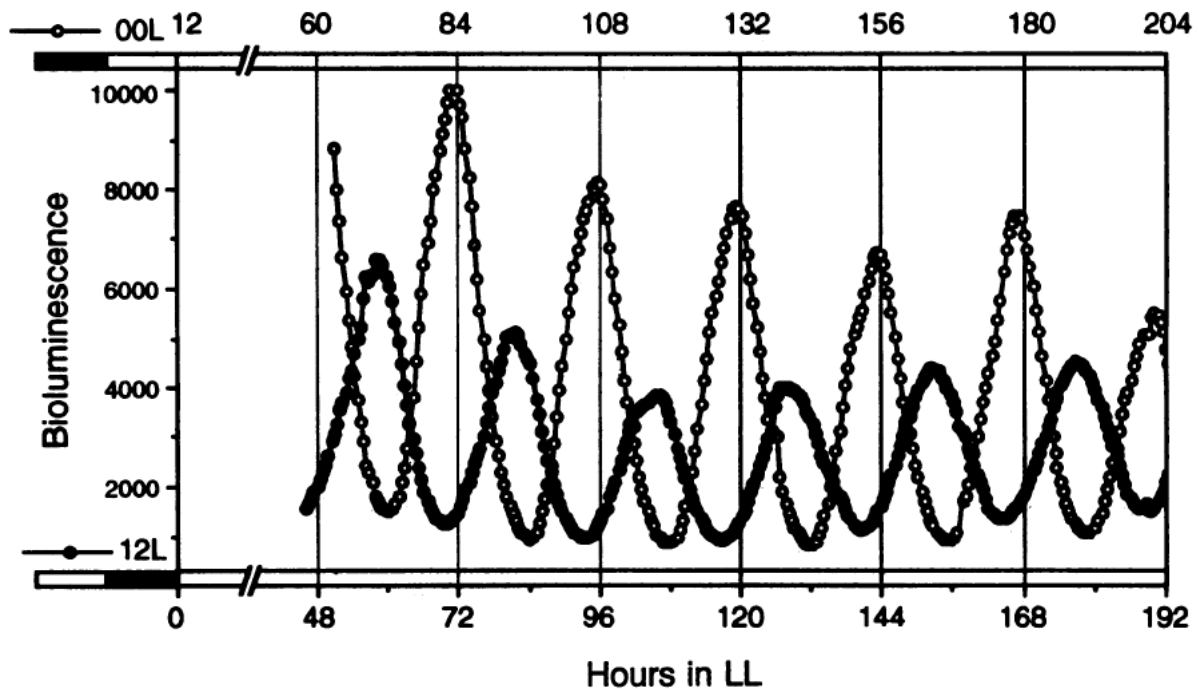


Figure1. Circadian rhythms of bioluminescence of *S.elongatus* PCC 7942 AMC149 strain in continuous light conditions.

Adapted from Kondo et al., PNAS, 1993. AMC149 strain contains a bioluminescence reporter *PpsbA1::luxAB* inserted in NS1. Cells were cultured at 30C and entrained with two LD cycles which were 12hr out of phase, represented by the open and closed circles. After released to constant light condition, the bioluminescence from both conditions oscillated with similar periods but with opposite phases.

### *Core clock genes in cyanobacteria*

This bioluminescence reporter enabled several major findings regarding circadian clocks in cyanobacteria, including the discovery of the core clock genes in 1998. Dr. Takao Kondo designed an automated turntable CCD-camera system to record in real-time the bioluminescent emission from single colonies on agar plates<sup>12</sup>. The invention of the “Kondotron” allowed a relatively high throughput screening of mutant strains, which were chemically mutagenized with ethyl methanesulfonate (EMS). Clock mutants that had arrhythmia or atypical periods were found<sup>13</sup>. These mutants could then be rescued by the introduction of a wild-type DNA library, which eventually resulted in the identification of the clock gene cluster *KaiABC*<sup>14</sup>. The *KaiA* promoter produces a monocistronic *KaiA* mRNA, whilst the *KaiB* promoter generates a dicistronic *KaiBC* mRNA<sup>14</sup>. Deletion of the entire *KaiABC* cluster or any of the three genes individually led to arrhythmia in bioluminescence traces<sup>14</sup>. A global circadian gene expression pattern was revealed as well in 1995 by a “promoter trap” study, in which a promoter-less *luxAB* gene was randomly inserted into the chromosome, suggesting that expression of all genes in *S.elongatus* might be under the control of the clock machinery<sup>15</sup>.

### *KaiABC system in cyanobacteria*

The phosphorylation state of KaiC oscillates robustly within the cells with a 24-hour period<sup>16</sup>. The KaiC protein has two critical phosphorylation sites, which are serine 431 (S431) and threonine 432 (T432)<sup>17,18</sup>. Single mutation of either of the phosphorylation sites on the KaiC protein leads to the disruption of the KaiC phosphorylation cycle and abolishes the rhythm *in vivo*<sup>17</sup>, indicating that KaiC

phosphorylation is necessary for pacemaker function in the cyanobacterial system<sup>17,18</sup>. KaiC has enzymatic activity in terms of autophosphorylation and autodephosphorylation at both sites. The KaiA protein enhances the phosphorylation of KaiC while KaiB antagonizes the activity of KaiA and thus promotes dephosphorylation of KaiC<sup>19-22</sup>.

Recently, remarkable progress has been achieved on the structures of KaiA, KaiB, and KaiC<sup>23</sup>. KaiC forms a hexameric complex with twelve ATP molecules bound at the interfaces between each subunit; every KaiC monomer consists of N-terminal (CI) and C-terminal (CII) halves that share similar conformations<sup>24</sup>. KaiA forms a dimer and the C-terminal portion of the protein seems to be essential for KaiA function. The isolated KaiA C-terminal portion has been shown to bind to KaiC, and each KaiA monomer dimerizes with another KaiA molecule to promote KaiC phosphorylation *in vitro*. The crystal structure of KaiB suggests that KaiB forms dimers or tetramers<sup>25</sup>. During the phosphorylation phase, KaiA interacts with the C-terminal tentacles of KaiC to enhance the autokinase activity. However, in the de-phosphorylation phase, KaiA is recruited by the KaiB\*KaiC complex to form a stable KaiA\*KaiB\*KaiC complex. This “recruitment” sequesters KaiA so that the free KaiA concentration in the reaction is decreased, thereby facilitating KaiC’s de-phosphorylation. It is believed that the association of KaiA with KaiC in the phosphorylation phase is labile because that the KaiA\*KaiC complex dissociates during electrophoresis through a native gel, while the KaiABC complex in the de-phosphorylation phase can resist dissociation by the native gel assay<sup>27</sup>.

### *Transcriptional and translational feedback loop (TTFL) model*

In eukaryotic organisms, the transcriptional and translational feedback loop (TTFL) model was proposed to explain the cyclic expression pattern of mRNAs and proteins encoded by clock genes<sup>28</sup>. This model explains that the protein products of the clock genes could negatively feedback on their own gene expression, which generates an auto-regulatory feedback loop. In *S.elongatus*, not only the transcripts of the KaiA and KaiBC<sup>14</sup>, but also the abundance of KaiB and KaiC proteins are rhythmic<sup>29</sup>. Overexpression of the *KaiA* gene causes an enhancement of *KaiBC* gene transcription, whilst *KaiC* overexpression represses it<sup>14</sup>. All these phenomena fit the TTFL model very well, by the interpretation that the clock genes are under negative regulation by the KaiC protein and positive regulation by the KaiA protein.

### *A post-translational oscillator (PTO) found in cyanobacteria*

It is known that *S.elongatus* is an autotrophic photosynthetic species; under constant dark condition (DD), most of the metabolic activities, including transcription and translation, are severely suppressed<sup>30</sup>. Surprisingly, the KaiC phosphorylation rhythm still persisted in DD<sup>29,30</sup>, which contradicts the TTFL model. So how does the KaiC phosphorylation rhythm remain in the absence of transcription and translation? In 2005, Nakajima et al. reported that the circadian oscillation in the phosphorylation level of KaiC can be reconstituted *in vitro* by incubating only KaiA, KaiB, KaiC and ATP<sup>20</sup>[figure2]. They discovered that this *in vitro* oscillator satisfies all three criteria of circadian clock: (1) it has a period of ~22 hours when incubated at 30°C, (2) is temperature compensated at different temperatures, and (3) it can also be reset by



temperature pulses. Most importantly, KaiC mutants, which exhibited a shortened or lengthened period *in vivo*, had a similar phenotype when reconstituted *in vitro*<sup>20</sup>. These findings have suggested that, unlike eukaryotes, the post-translational oscillator (PTO) might function as the core clock machinery in cyanobacteria.

Circadian systems are comprised of at least three elements: a) input pathways, which convey external information including time of day to a central oscillator, b) a central oscillator that generates the rhythm, and c) output pathways, through which the oscillator regulates gene expressions and biological processes. The *in vitro* oscillator in cyanobacteria enables us to investigate the central oscillator directly without interference from input or output pathways and as a result, is the best model so far to study the molecular mechanisms underlying a circadian biological clock.

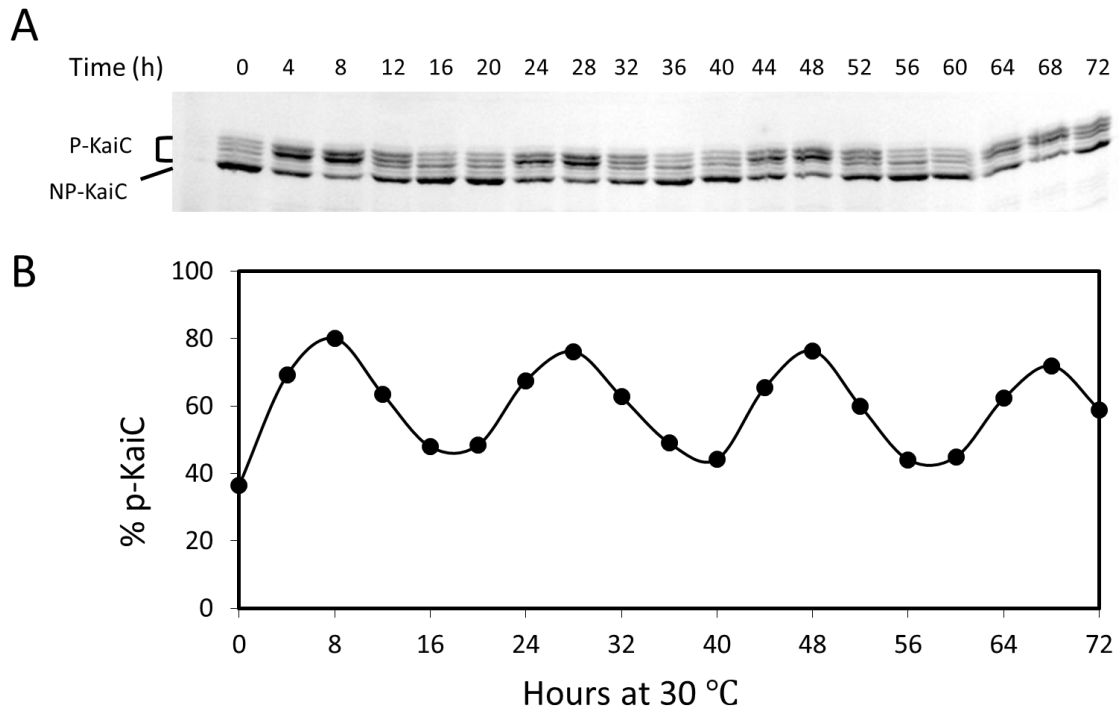


Figure2. *In vitro* oscillation of KaiC phosphorylation states.

(A) KaiC proteins were incubated with KaiA and KaiB in the presence of ATP. A time-course reaction shows a robust KaiC phosphorylation rhythm resolved by SDS-PAGE gel. The upper bands correspond to phosphorylated KaiC (P-KaiC) and the bottom band is the non-phosphorylated KaiC (NP-KaiC). (B) A quantification of the SDS-PAGE image. The ratio of P-KaiC to total KaiC was plotted as the function of incubation time.

## CHAPTER II

### SYNCHRONIZATION OF OSCILLATORS WITH DIFFERENT PERIODS *IN VITRO* AND *IN VIVO*

#### Introduction

Robust, sustained oscillation in constant conditions with a ~24-hour period is a canonical characteristic of circadian rhythms. Even in cyanobacteria, which lack cell-cell communication according to our current understanding, the circadian clocks among cells in a population remain precisely in synch. A single cell's oscillator is under constant noise as well, due to cellular transcription and translation. These indicate that multiple KaiC complexes are capable of staying sync with one another. This hypothesis has been partly demonstrated by the observation that the *in vitro* oscillation persists more than 10 days without damping<sup>31</sup>. Strong evidence is provided by Ito et al. in 2007<sup>31</sup>. They first investigated the synchronization of oscillations between different phases of KaiC. A sample which is in the dephosphorylation phase can shift the phase of other reaction samples via alteration of the phosphorylation direction of the second sample. In this case, the second sample changes from phosphorylation to dephosphorylation, bringing the phase of the latter sample into alignment with the former <sup>31</sup>[Figure 3A]. What causes this synchronization remains elusive, however, researchers have come up with two hypotheses: the so-called "KaiC monomer exchange" and the "KaiA sequestration" model<sup>32</sup>.

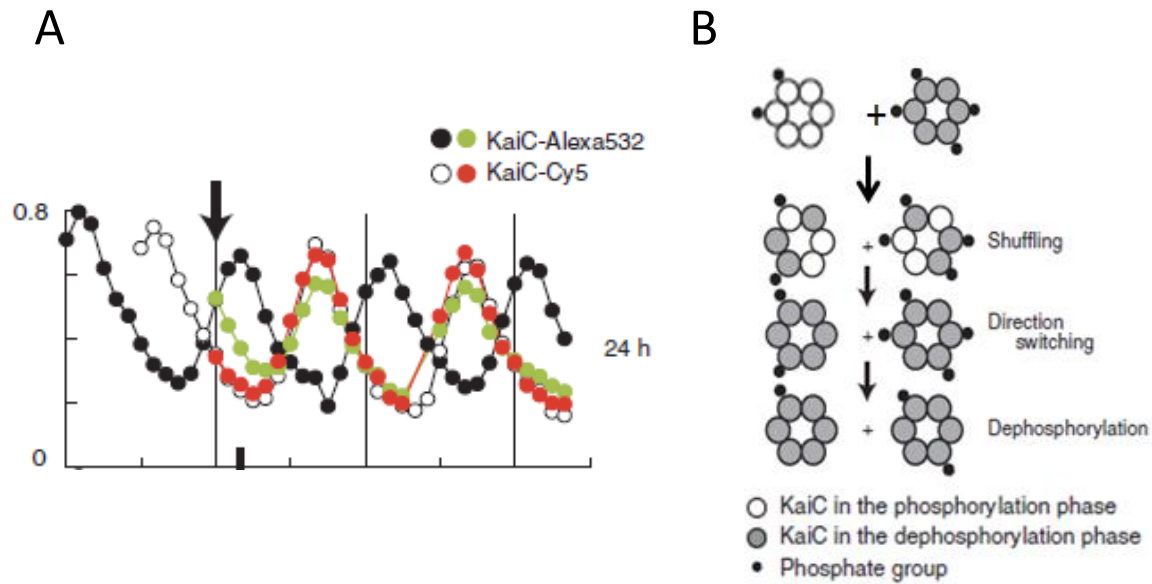


Figure3. Synchronization of KaiC phosphorylation rhythms with different phases. Adapted from Ito et al., 2007. (A) Synchronization of oscillations with different phases reported by Ito et al. (2007). KaiC proteins from the sample that was originally in the phosphorylation phase underwent dephosphorylation upon mixing with sample in dephosphorylation phase<sup>31</sup>. (B) “Monomer exchange” model to explain the synchronization phenomena. KaiC monomers within a hexamer that are in phosphorylation phase will switch to dephosphorylation phase when monomers in dephosphorylation phase are integrated into the hexamer<sup>31</sup>.

The “KaiC monomer exchange” model suggested that KaiC monomers can shuffle between two KaiC hexamers synchronization<sup>31,33,34</sup>. The reasons that KaiC monomer exchange can promote synchronization are that (1), it could bring equalization of the phosphorylation states among KaiC hexamers in the population, and (2) KaiC monomers that are in phosphorylation phase tend to switch to dephosphorylation when monomers from another KaiC hexamer, which is in dephosphorylation phase, are integrated [Figure 3B]. The phenomenon of KaiC monomer exchange was first reported using a pull-down assay<sup>34</sup>. Flag-tagged and His6-tagged KaiC were mixed, incubated, and pulled down by the antibody against Flag. Monomer exchange between two KaiC hexamers was claimed based on the fact that a greater number of KaiC hexamers could be immunoprecipitated by anti-FLAG beads several hours after mixing compared to time 0. They also reported that the rate of monomer exchange appeared to be maximal during the phase of KaiC dephosphorylation. KaiC monomer exchange was also detected by fluorescence resonance energy transfer (FRET). The FRET signal was observed when mixing two groups of KaiC, which were labeled with a pair of fluorophores, IAEDANS and MTSF, respectively<sup>33</sup>. Furthermore, mathematical modeling was widely used to investigate monomer exchange. Mori et al. (2007) showed that when monomer exchange was allowed, the *in vitro* rhythm of KaiC phosphorylation was well sustained, but when monomer exchange was disallowed, it would dampen rapidly<sup>33</sup>.

In contrast, the “KaiA sequestration” model was also proposed by a different group<sup>32</sup>. Mathematical modeling suggested that KaiB proteins could bind the hyperphosphorylated form of KaiC and the resulting KaiBC complex could then recruit, bind, and thus sequester free KaiA dimers, which stabilized KaiC in a default

dephosphorylating state<sup>32</sup>. Later that year, a paper from the O'Shea lab strongly supported this model experimentally. They introduced dephosphorylated KaiC into the reaction at various time points to indicate that KaiA was only active during the phosphorylation phase of the oscillator, since the added KaiC became phosphorylated only when they were added during the phosphorylation phase of the oscillator. This paper confirmed that each cycle of the oscillator was composed of a phosphorylation phase of high KaiA activity, followed by a dephosphorylation phase during which free KaiA proteins were sequestered with KaiBC into a KaiABC complex and became inactive<sup>35</sup>. Subsequently, native gel assays were used to show that a stable KaiA\*KaiB\*KaiC complex rhythmically assembled in antiphase to the KaiA dimer bands during the oscillation<sup>27</sup>. To explain the results of the Ito et al. paper<sup>31</sup> for this model, the KaiABC complex in the de-phosphorylation phase is capable of recruiting free KaiA molecules from samples in other phases (e.g., phosphorylation phase) and thereby inducing the phase-switch of the latter sample from phosphorylation to dephosphorylation.

## Results

### *Synchronization of oscillators with different periods in vivo*

Synchronization of *in vitro* oscillators in different phases has been confirmed and investigated<sup>31</sup>. Their discovery brought another interesting question: whether oscillators with different periods could synchronize. Many cyanobacteria, including the species used in our study contain multiple copies of the KaiABC gene cluster due to multiple, identical copies of chromosomes per cell; therefore, it is interesting to know whether they will show abnormal circadian rhythms/ behavior if two oscillators with different periods coexist due to mutations of *KaiC* gene, which is an essential cog in the cyanobacterial circadian clock machinery.

A number of mutations in the *KaiC* gene shorten or extend the period of circadian rhythms in *S. elongatus*. For example, bioluminescence rhythms *in vivo* (KaiBC promoter; luxAB reporter) show period lengths of 20 & 28 hours, respectively, in mutant strains with single amino acid substitutions T415A and T42S, compared to the KaiC<sup>WT</sup> strain with a period ~25hours. We obtained the pKaiABC-NSII plasmid from Dr. Xu. This plasmid was designed to put an entire KaiABC locus under the control of its endogenous promoter to NSII in the cyanobacterial genome. We mutated the WT *KaiC* in the plasmid to *KaiC*<sup>T42S</sup> or *KaiC*<sup>T415A</sup> by site directed mutagenesis while leaving *KaiA* and *KaiB* intact. The mutated plasmid pKaiABCT42S-NSII, pKaiABCT415A-NSII or pKaiABC-NSII (control) was transformed to the AMC149 strain, which is basically a wild-type strain with a psbAI::luxAB reporter in NSI [Figure4]. Therefore, our new

strains presumably contained two oscillators per cell: one driven by KaiC<sup>WT</sup> and the other by a mutant version of KaiC.

The bioluminescence of each strain emitted from *psbA1::luxAB* was monitored by the Kondotron in real-time under constant light condition at 30°C. Representative examples are shown in figure 5. As expected, all strains exhibited robust rhythms similar to the wild type strain AMC149, which suggested that different oscillators coexisting in a single cell will not cause arrhythmia or other abnormal clock patterns including bimodal. The strain from the control plasmid (KaiABC<sup>WT</sup>-AMC149) showed a slightly shorter period, whilst KaiABC<sup>T42S</sup>-AMC149 and KaiABC<sup>T415A</sup>-AMC149 exhibited a significantly longer and shorter period, respectively. Quantification of all the recorded strains is shown in Figure 6A. KaiABC<sup>T42S</sup>-AMC149 strain exhibited an intermediate period compared to KaiABC<sup>T42S</sup> (~28h) and AMC149 (~24.7h), and similarly, KaiABC<sup>T415A</sup>-AMC149 displayed an intermediate period as well.

An even shorter-period KaiC mutant (KaiC<sup>F470Y</sup>) was also tested to confirm our findings. This strain was reported in 2005<sup>20</sup>, which had a robust short-period rhythm of ~17h both *in vivo* and *in vitro*. Therefore, it will be even harder for them to synchronize since they have an 8h-period difference. We were surprised to observe that KaiABC<sup>F470Y</sup>-AMC149 displayed a robust rhythm with non-reduced amplitude similar to AMC149 as well. After quantification of periods, again we found this strain showed an intermediate period (~20h) compared to KaiABC<sup>F470Y</sup> (17h) and AMC149 [Figure 7A].



### *Period changes according to different expression levels of KaiC mutants*

We have demonstrated that coexpressing two oscillators with different periods led to a robust rhythm of an intermediate period. To further investigate this phenomenon, we want to know whether increased expression levels of *KaiC* mutant gene will “push” the resulting rhythm closer to the mutant, in a co-expression *in vivo* experiment.

We were planning to put all three *KaiA* *KaiB* and *KaiC* genes under the control of the *P<sub>trc</sub>* promoter, whose expression level can be manipulated by addition of IPTG, in order to maintain the normal stoichiometry of Kai genes. However, due to technical difficulties we elected to simplify the experimental design and perform the co-expression experiment with *KaiC* only. Though expression of *P<sub>trc</sub>::KaiC* in AMC149 will result in a change of stoichiometry of *Kai* genes, the results will be easier to interpret. The mutated plasmids *P<sub>trc</sub>::KaiC<sup>T42S</sup>-NSII* or *P<sub>trc</sub>::KaiC<sup>T415A</sup>-NSII* or *P<sub>trc</sub>::KaiC-NSII* (control) were transformed to AMC149 respectively. Our new strains contained one endogenous *KaiABC* gene, and another copy of inducible mutated *KaiC*.

We monitored the bioluminescence rhythm of each strain treated with increasing amounts of IPTG in succession (0uM, 0.5uM, 1uM, 2uM, 5uM) with the Kondotron. Quantification is depicted in Figure 6B. The AMC149 (WT) strain maintained approximately the same period over the increasing concentration of IPTG, whereas *P<sub>trc</sub>::KaiC-AMC149* slightly lengthened its free running period due to changed stoichiometry of Kai proteins in cells. *P<sub>trc</sub>::KaiC<sup>T42S</sup>-AMC149* displayed a longer period that ranged from 26 to 28 hrs depending on the IPTG level and correspondingly the level of *KaiC<sup>T42S</sup>* protein in the cells. On the contrary, *P<sub>trc</sub>::KaiC<sup>T415A</sup>-AMC149* showed a

slightly shorter period when IPTG increased but the effect was not statistically significant [Figure 6].

Following the previous experiment, Ptrc::KaiC<sup>F470Y</sup>-AMC149 was constructed and tested as well. Surprisingly, this strain's period was affected by increasing IPTG concentration: the free running period dropped from 22 to 20hr [Figure 7B].

Our results have suggested that addition of KaiC proteins in cyanobacteria did not lead to arrhythmia or other abnormal phenotypes, even though the extra copy of KaiC protein was a period mutant. For instance, co-expression of KaiC<sup>WT</sup> and short-period mutant KaiC<sup>F470Y</sup> generated a perfectly synchronized rhythm pattern without bimodal phenomenon. Additionally, up-regulated KaiC<sup>F470Y</sup> proteins seem to be able to “kidnap” the overall period; in other words, the resulting rhythm has a period more like the short-period mutant.

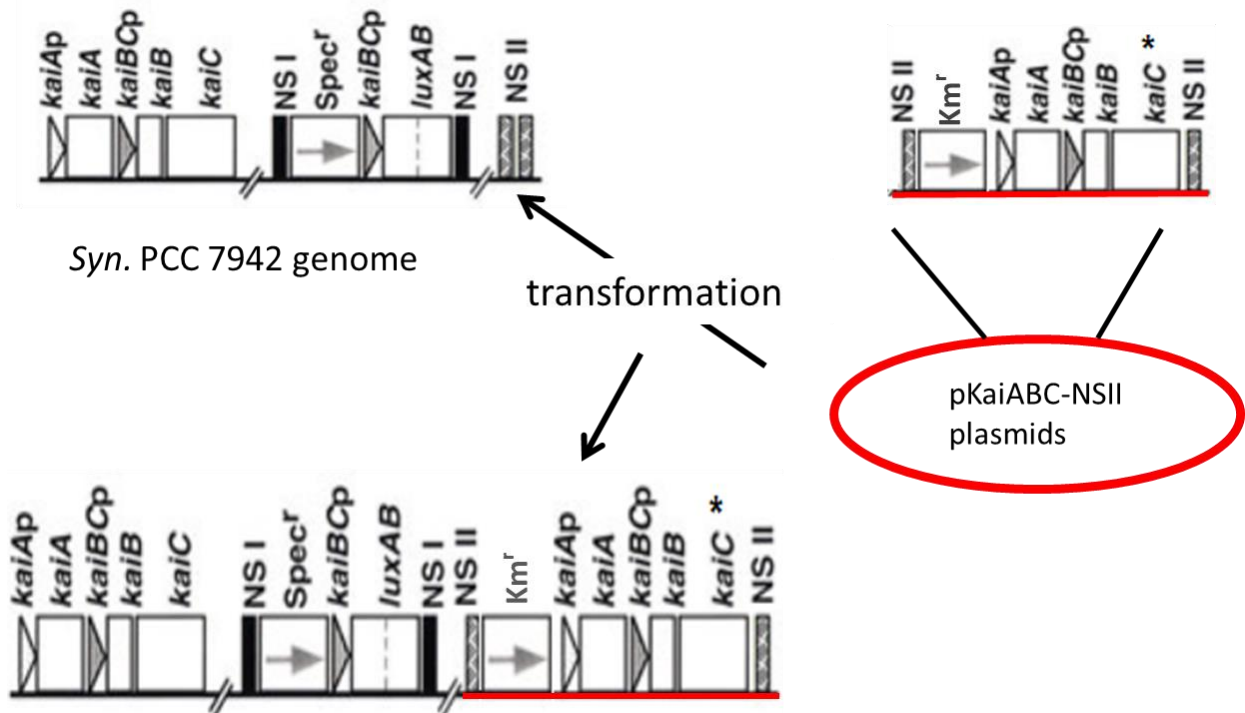
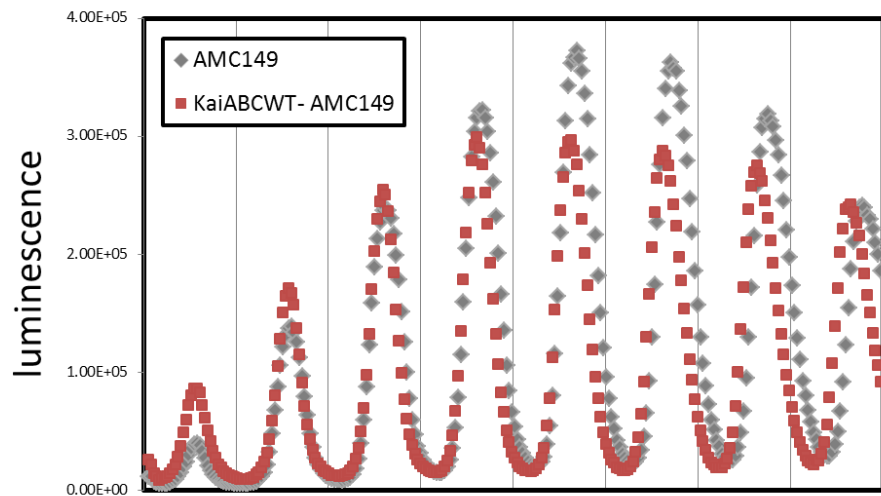
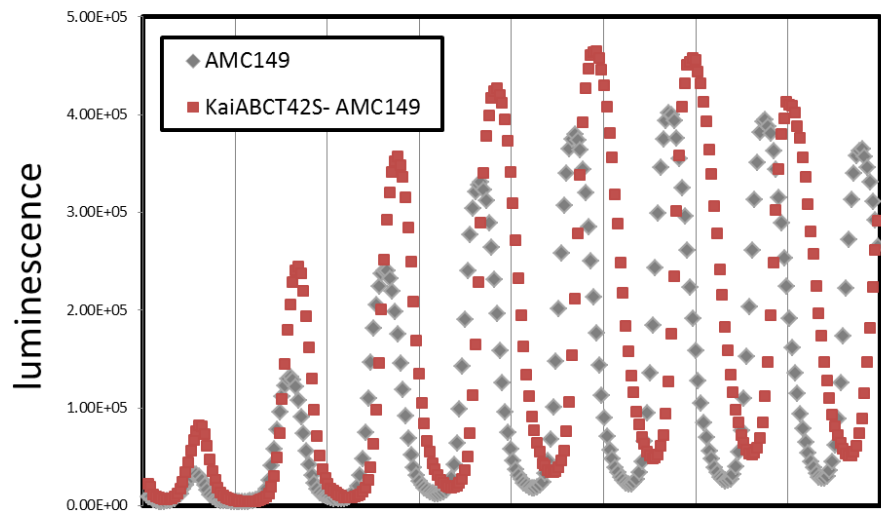


Figure 4. A generalized diagram for the strains used in the study. Three loci in the cyanobacterial chromosome are shown here: (1) the endogenous KaiABC cluster; (2) the *KaiBC::luxAB* or *psbA1::luxAB* reporters in neutral site 1 (NSI); and (3) another KaiABC cluster inserted in the neutral site 2 (NSII), shown as the red line. *Km<sup>r</sup>*, kanamycin resistance gene; The *KaiC\** in pKaiABC-NSII plasmids stand for different *KaiC* period mutations conferring long-period (*KaiCT42S*) and short-period (*KaiCT415A* and *KaiCF470Y*).

A



B



C

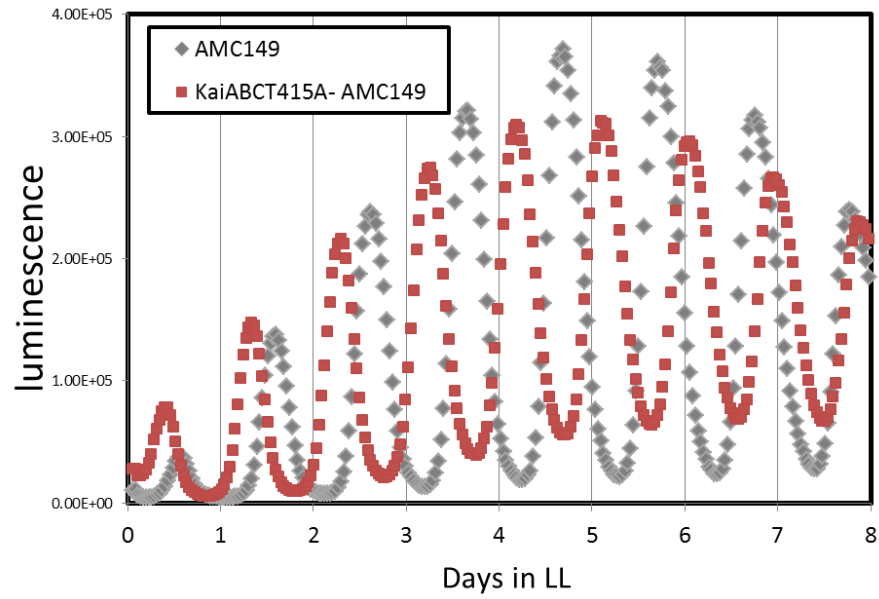


Figure 5. Synchronization of oscillators with different periods *in vivo*.

Co-expression of two oscillators with different periods by inserting another copy of KaiA, KaiB and KaiC\* (stands for KaiC<sup>WT</sup>, KaiC<sup>T42S</sup> or KaiC<sup>T415A</sup>) into NSII in chromosomes of AMC149 (wildtype). (A) One representative example is shown here to indicate that co-existing of WT with WT oscillator leads to a robust rhythm with a slightly short period (red trace), compared to AMC149 itself (grey). (B) Co-existing long-period (KaiC<sup>T42S</sup>) and WT oscillators synchronize *in vivo* and lead to an overall robust rhythm but with a significantly lengthened period (red trace), compared to AMC149 itself (grey). (C) Co-expression of short-period (KaiC<sup>T415A</sup>) with WT oscillator results in an oscillation with a shortened period.

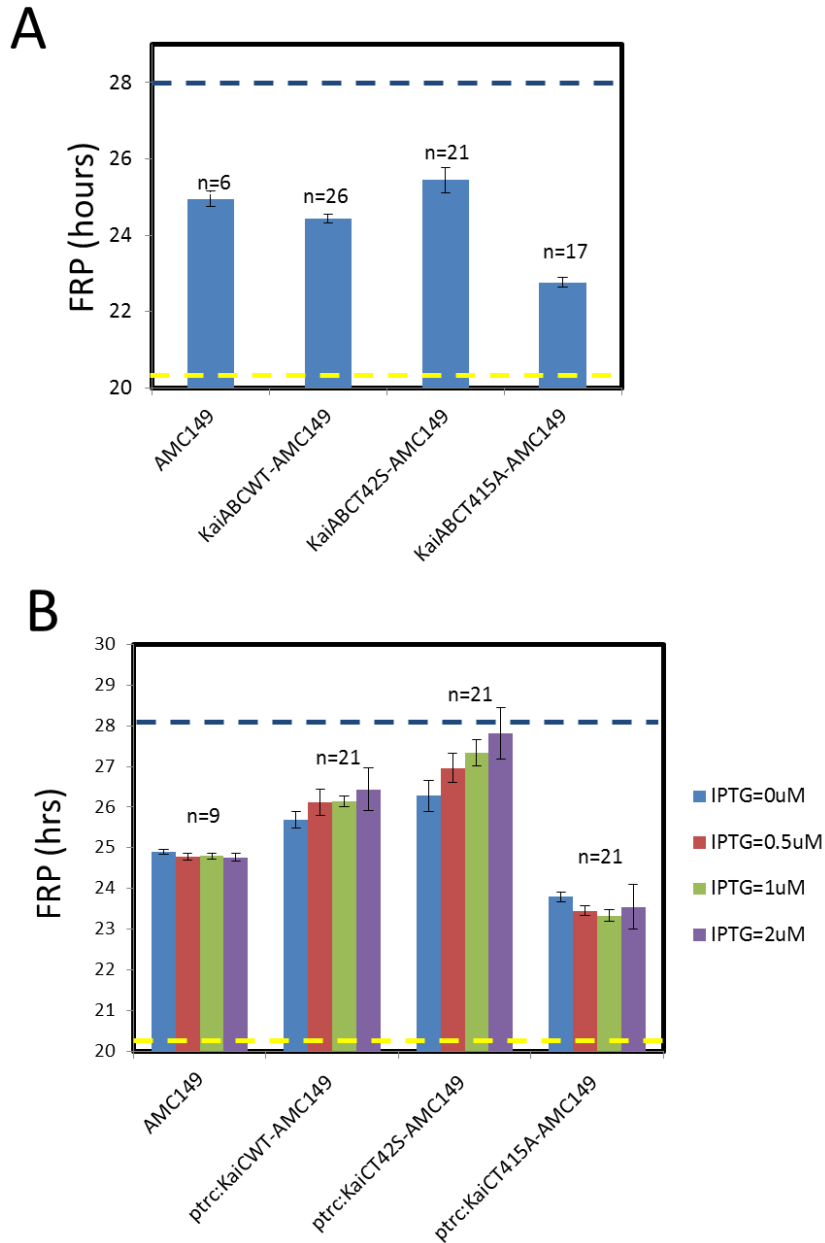


Figure 6. Quantification of the synchronization experiments (WT, T42S and T415A) *in vivo*.

(A) Co-expression of two oscillators with different periods reveals a synchronized rhythm and the resulting period for each co-expression strain is analyzed and plotted. Blue line is the period for long-period mutant ( $KaiC^{T42S}$ ) alone, and the yellow line is the period for short-period mutant ( $KaiC^{T415A}$ ) itself. Each co-expression strain displays an intermediate period. (B) Increased expression levels of *KaiC* mutant genes by IPTG will “push” the resulting rhythm to possess a period more like the mutant, in a co-expression *in vivo* experiment. Blue line is the period for long-period mutant ( $KaiC^{T42S}$ ) alone, and the yellow line is the period for short-period mutant ( $KaiC^{T415A}$ ) itself.

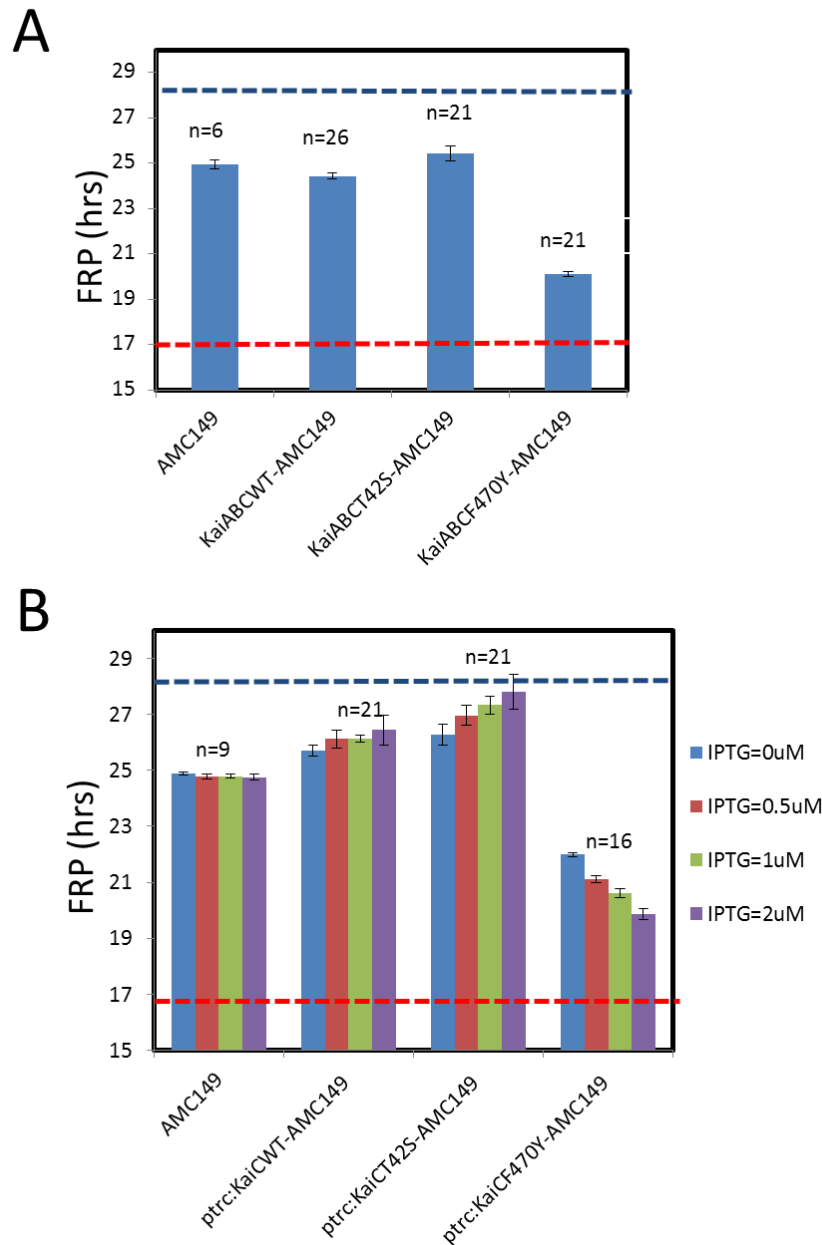


Figure 7. Quantification of the synchronization experiments (WT, T42S and F470Y) *in vivo*.

(A) Co-expression of two oscillators with different periods reveals a synchronized rhythm and the resulting period for each co-expression strain is analyzed and plotted. Blue line is the period for long-period mutant (KaiC<sup>T42S</sup>) alone, and the red line is the period for short-period mutant (KaiC<sup>F470Y</sup>). Each co-expression strain displays an intermediate period. (B) Increased expression levels of *KaiC* mutant genes by IPTG will “push” the resulting rhythm to possess a period more like the mutant, in a co-expression *in vivo* experiment. Blue line is the period for long-period mutant (KaiC<sup>T42S</sup>) alone, and the yellow line is the period for short-period mutant (KaiC<sup>F470Y</sup>).

### *Synchronization of oscillators with different periods in vitro*

Co-expression experiments *in vivo* have partially answered whether oscillators with different period could synchronize, but do not fully solve the problem. For example, does the transcription and translation play a role in facilitating the synchronization of different oscillators? Since KaiC proteins are hexamers, when they are translated as monomers, are the KaiC<sup>WT</sup> and KaiC mutant monomers recruited together as chimeras? If not, will KaiC<sup>WT</sup> and mutant proteins undergo monomer exchange over time? Fortunately, the cyanobacterial circadian clock is the only model clock to have been reconstituted *in vitro*, which enables us to investigate the central oscillator directly without interference from input and output pathways.

To analyze the phosphorylation status of KaiC mutants versus KaiC<sup>WT</sup> in SDS-PAGE gels, I genetically attached a 3Myc tag to the N-terminal of KaiC<sup>WT</sup>. I used the N-terminals because the C terminal tentacles are vital for interaction with KaiA<sup>36</sup>. The addition of the 3Myc tag did not affect the activity of KaiC, and the phosphorylation rhythm *in vitro* was unaffected with a period of 20 hours, which is comparable to KaiC<sup>WT</sup> proteins *in vitro* [Figure 8B,8C]. Moreover, the addition of the 3Myc tag has allowed a visible band shift to permit detection of the respective phosphorylation states of WT KaiC and 3Myc-KaiC [Figure8A].

I introduced a single amino acid mutation to *KaiC* in the pGEX-6p-1-KaiC plasmid, which is a GST gene fusion vector designed to affinity purify proteins with the GST tag, to make constructs for purifying KaiC<sup>T42S</sup> and KaiC<sup>T415A</sup> proteins. The two proteins were then expressed in *E.coli*, pulled down by glutathione beads, and further purified by ion exchange chromatography. The two KaiC mutant proteins possessed a comparable



molecular size as that of WT KaiC. We expected that the KaiC<sup>T42S</sup> protein should have a longer period upon incubation with KaiA, KaiB and ATP, since it exhibits a lengthened period *in vivo*. Likewise, KaiC<sup>T415A</sup> was expected to display a short *in vitro* rhythm. In Figure 9, as expected, the amplitude of the phosphorylation state of KaiC<sup>T42S</sup> oscillated between minimal (20%) and maximal (80%) values robustly over time with a longer period (24hr) compared to 3myc-KaiC [Figure 8B, 8C, 9A, 9B]. On the contrary, the phosphorylation state of KaiC<sup>T415A</sup> stayed the same over time (data not shown), i.e., it did not oscillate. We tried to re-purify and re-test the protein several times, but the same result was obtained.

We have subsequently tried several different short-period mutants, including KaiC<sup>P248A</sup>. This mutant is a temperature-dependent mutant; the period varies over the spectrum of temperature *in vivo*. In this case, the strain's period is shortened as the temperature increases ( $Q_{10}=1.3$ ) (unpublished data of Dr. Mori). The *in vitro* activity revealed that this mutant did have a robust short period (17.5hr) at 30°C [Figure 11A, 11B].

To test the synchronization of oscillators with different periods *in vitro*, we first dephosphorylated all the KaiC proteins for 24 hours separately. Two reactions (3myc-KaiC+KaiC<sup>T42S</sup> or 3myc-KaiC+KaiC<sup>P248A</sup>) were mixed and initiated by addition of 1 mM ATP the following day. In the reaction comprised of 3myc-KaiC and KaiC<sup>T42S</sup>, robust KaiC phosphorylation rhythms for both KaiCs were resolved by SDS-PAGE gel [Figure10A]. A quantification of the SDS-PAGE gel is shown in Figure 10B. Two KaiC populations oscillated together with a shared period, and no obvious phase differences could be detected. Additionally, the mixture exhibited an intermediate period (22hr)

between 3myc-KaiC (20hr) and KaiC<sup>T42S</sup> (24hr), when we compared the phosphorylation state at each time point.

However, the same result was not obtained in the case of 3myc-KaiC and KaiC<sup>P248A</sup>. The result in Figure 12 showed that after mixing, both oscillators exhibited severe damping amplitude. Though it still appeared to have a shared period (approximately 18.5hr) and phase [Figure 12B], it remains inconclusive explicitly due to the difficulty of distinguishing phases of the rhythms due to reduced amplitude.

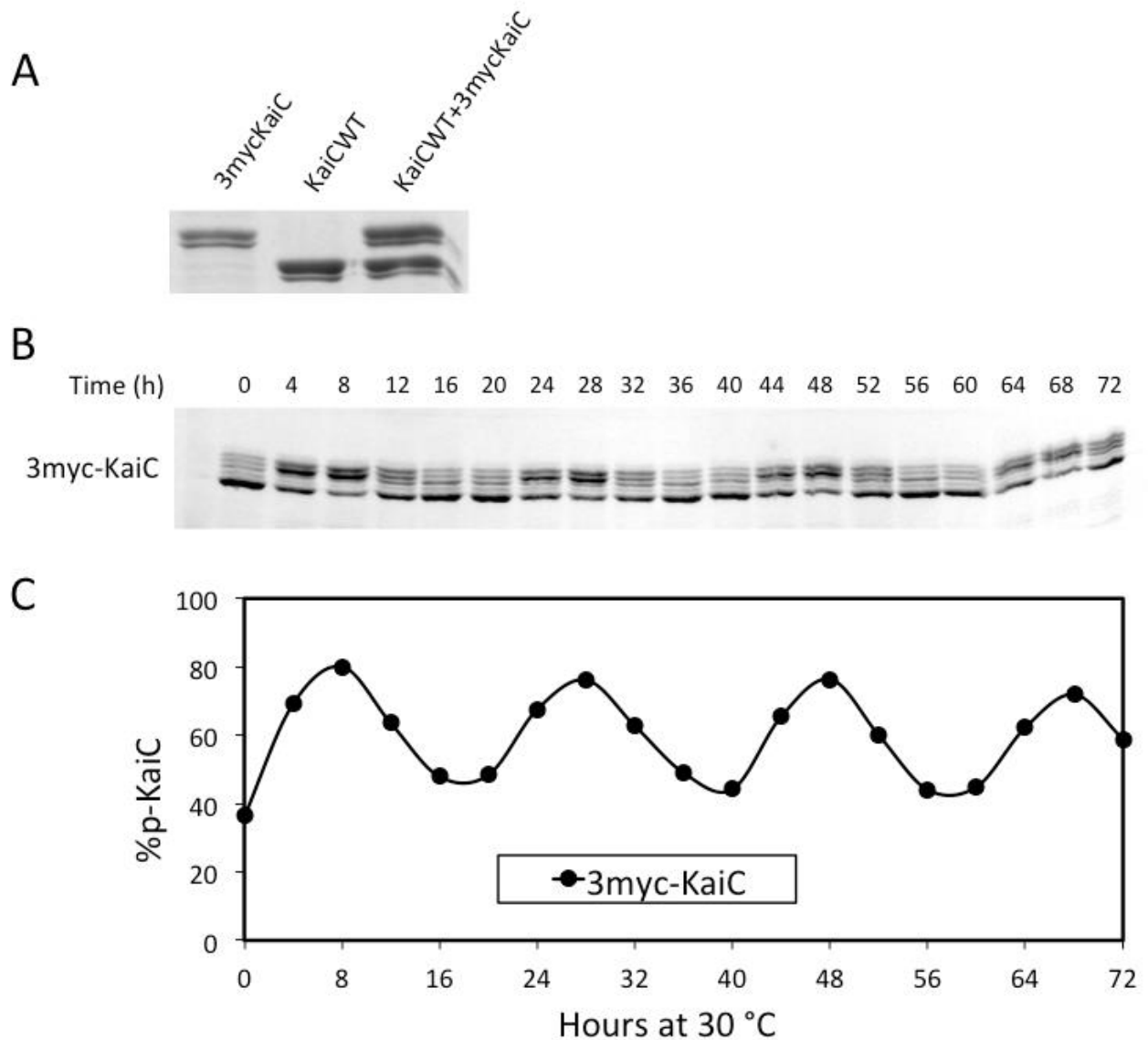


Figure 8. 3Myc tagged KaiC retained a strong *in vitro* phosphorylation rhythm similar to WT KaiC.

(A) 3Myc tagged KaiC was purified in order to introduce enough gel shift to resolve tagged KaiC and KaiC period mutants in the following synchronization experiments. (B) 3myc-KaiC proteins were incubated with KaiA and KaiB in the presence of ATP. A time-course reaction shows a robust KaiC phosphorylation rhythm resolved by SDS-PAGE gel. (C) A quantification of the SDS-PAGE image. The ratio of P-KaiC to total KaiC was plotted as the function of incubation time. 3myc tagged KaiC retained the activity to oscillate robustly with a period just like WT KaiC.

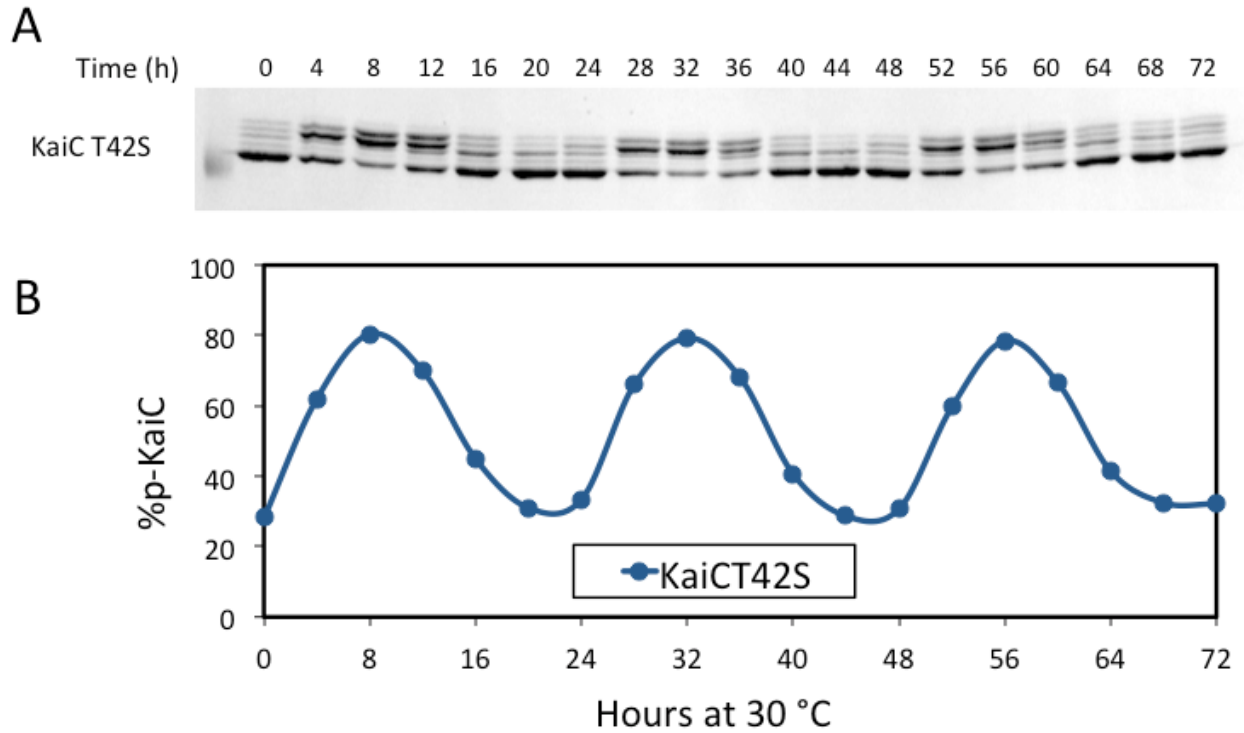


Figure 9. KaiC<sup>T42S</sup> mutant possessed a strong *in vitro* phosphorylation rhythm with a long period.

(A) KaiC<sup>T42S</sup> proteins were incubated with KaiA and KaiB in the presence of ATP. A time-course reaction shows a robust KaiC phosphorylation rhythm resolved by SDS-PAGE gel. (C) A quantification of the SDS-PAGE image. The ratio of P-KaiC to total KaiC was plotted as the function of incubation time. KaiC<sup>T42S</sup> mutant has a robust oscillation but with an obvious long period compared to WT KaiC.

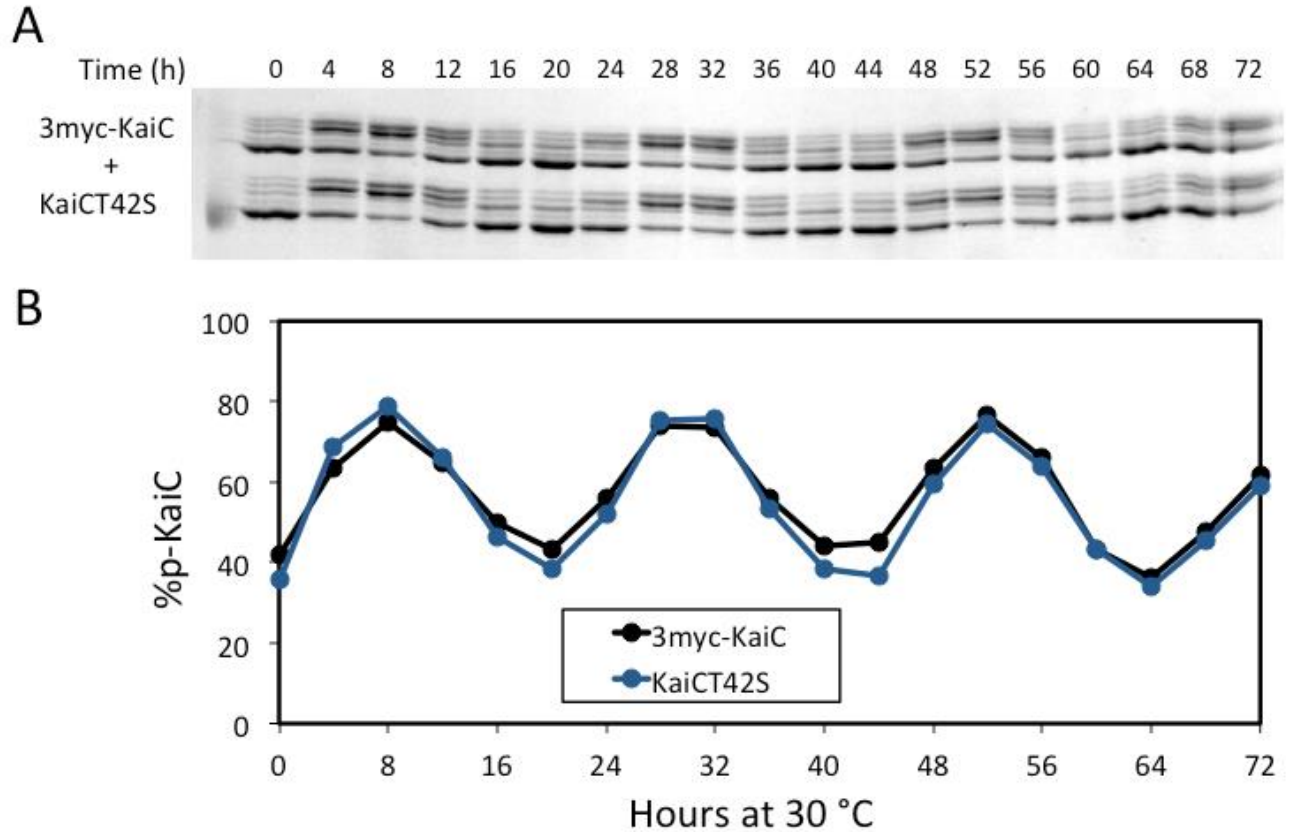


Figure 10. Two oscillators (3myc-KaiC and KaiC<sup>T42S</sup>) with different periods synchronized with a shared period and phase.

(A) KaiC<sup>T42S</sup> and 3myc-KaiC proteins were mixed at time point 0, and incubated with KaiA and KaiB in the presence of ATP. A time-course reaction showed robust KaiC phosphorylation rhythms for both KaiCs resolved by SDS-PAGE gel. The upper bands (larger size) correspond to 3myc-KaiC due to extra molecular weight and charge and the bottom bands are KaiC<sup>T42S</sup> mutant proteins that have similar molecular weight as WT KaiC. (B) A quantification of the SDS-PAGE image. The ratio of P-KaiC to total KaiC for both KaiC proteins was plotted as the function of incubation time separately. Two KaiCs synchronized together with a shared period and phase.

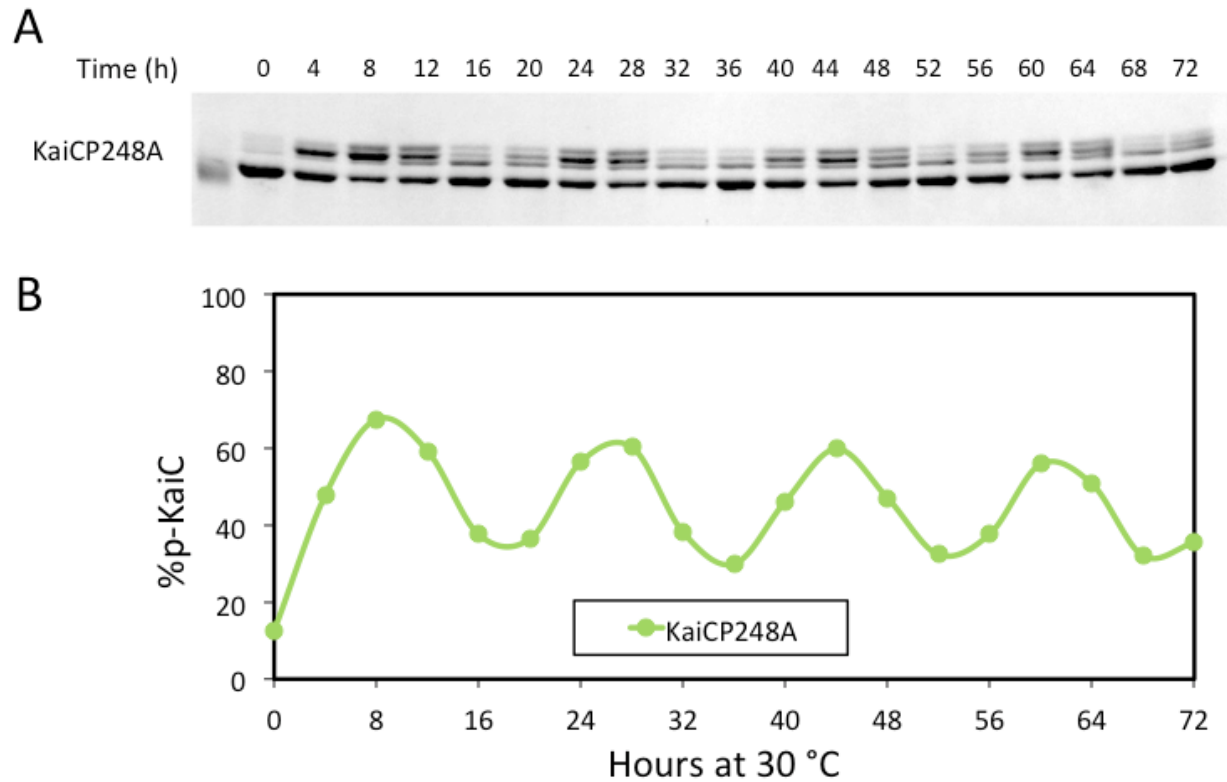


Figure 11. KaiC<sup>P248A</sup> mutant possessed a strong *in vitro* phosphorylation rhythm with a short period.

(A) KaiC<sup>P248A</sup> proteins were incubated with KaiA and KaiB in the presence of ATP. A time-course reaction shows a robust KaiC phosphorylation rhythm resolved by SDS-PAGE gel. (C) A quantification of the SDS-PAGE image. The ratio of P-KaiC to total KaiC was plotted as the function of incubation time. KaiC<sup>P248A</sup> mutant has a robust oscillation but with an obvious long period compared to WT KaiC.

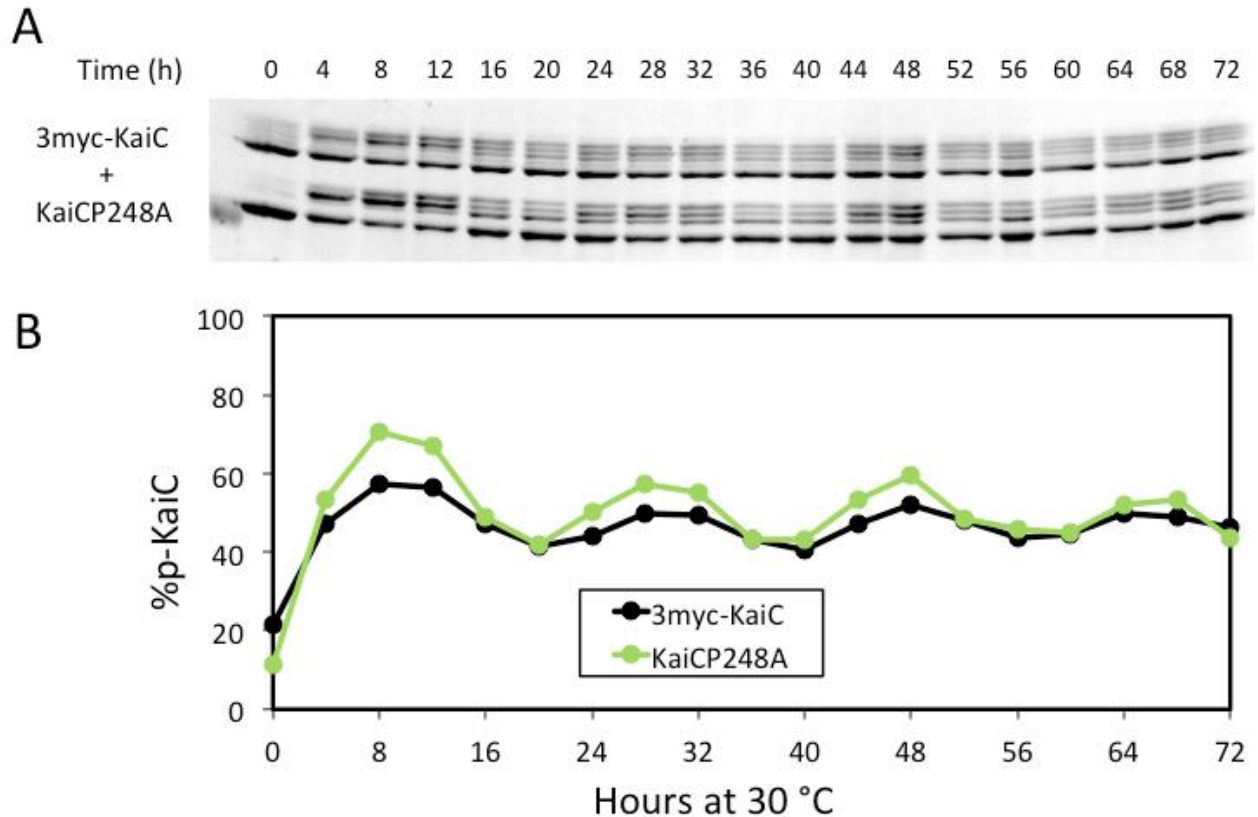


Figure 12. Two oscillators (3myc-KaiC and KaiC<sup>P248A</sup>) with different periods synchronized with a shared period and phase, but showed dampened rhythms. (A) KaiC<sup>P248A</sup> and 3myc-KaiC proteins were mixed at time point 0, and incubated with KaiA and KaiB in the presence of ATP. A time-course reaction showed robust KaiC phosphorylation rhythms for both KaiCs resolved by SDS-PAGE gel. The upper bands (larger size) correspond to 3myc-KaiC due to extra molecular weight and charge and the bottom bands are KaiC<sup>P248A</sup> mutant proteins that have similar molecular weight as WT KaiC. (B) A quantification of the SDS-PAGE image. The ratio of P-KaiC to total KaiC for both KaiC proteins was plotted as the function of incubation time separately. Two KaiCs synchronized together with a shared period and phase, but the rhythms dampened over time.

### *Computational simulation of synchronization of oscillators with different period*

Ito et al. reported synchronization of samples in different phosphorylation phases could be achieved with a synchronized, shared phase. And our work here partially demonstrated that oscillators with different period oscillators could synchronize together both *in vivo* and *in vitro*. Will computational simulation successfully predict the synchronization phenomena? Mori et al. reported a matrix model to perform computational simulation of the *in vitro* KaiABC oscillator<sup>33</sup>. Their model successfully predicted the dynamics of KaiABC complexes and emphasized the important role of monomer exchange. The advantage of this model is that it enables us to track the dynamics of every hexamer, even each monomer, which is helpful to simulate synchronization experiments since we need to keep track of two populations of KaiC separately during the entire simulation.

To model the synchronization of oscillators with different periods *in vitro*, we adopted the matrix model. The period of sustained oscillations is relatively insensitive to the on/off rate of KaiB-KaiC interaction, relaxation rate and monomer exchange rate. However, there is a strong dependence of the period of oscillation on the KaiA-KaiC phosphorylation rate (Type1), KaiC\* (a different KaiC conformation) de-phosphorylation rate (Type2), and on/off rate of KaiA-KaiC interaction (Type3). A change in the rate of the KaiA-KaiC phosphorylation rate (Type1), including a decrease (1.69E-04 to 0.84E-04) or increase (1.69E-04 to 2.53E-04), will significantly lengthen or shorten the period of oscillation, respectively [Figure 13B, 14B]. A similar result was acquired when we decreased or increased the possibility of KaiC\* de-phosphorylation rate (Type2) [Figure 15B, 16B], and the on/off rate of KaiA-KaiC interaction (Type3) [Figure 17B, 18B].



We modified the program so that we can track the origin of every monomer in the mixture of two KaiC populations with different periods. Additionally, we calculated an overall rhythm of KaiC phosphorylation status and rhythms for both KaiC populations separately.

We first simulated synchronization of oscillators between KaiC<sup>WT</sup> and a long-period KaiC which carried a type1 mutation (i.e. a slower rate of KaiC phosphorylation). The overall KaiC phosphorylation rhythm after mixing the two oscillators is shown in Figure 13C. It displays a robust rhythm with non-reduced amplitude similar to a single oscillator with an intermediate period. To figure out how this is achieved, we track each KaiC population, shown in Figure 13D. KaiC<sup>WT</sup> slightly lengthens its period while long-period KaiC shortens its period which leads to a sync of both the period and the phase.

Does monomer exchange play a role in the synchronization? Monomer exchange is deemed as the basis for equalizing dephosphorylation rates among KaiC hexamers<sup>31</sup>, and recent work also suggests that the KaiC phosphorylation oscillation will dampen without monomer exchange<sup>33</sup>. In our synchronization simulations, when monomer exchange is disallowed, the overall rhythm of KaiC phosphorylation dampens rapidly compared to when ME is allowed [compare Figure13 C with Figure 13 E]. Therefore, they oscillate with different frequencies and stay because the long-period and WT oscillators fail to synchronize after mixing. The end result is that the mixed-population oscillator will dampen rapidly in the absence of monomer exchange [Figure 13F].

Additionally, we also simulated synchronization of oscillators between KaiC<sup>WT</sup> and a short-period KaiC which carried a type1 mutation and the result is the same: the two oscillators synchronize perfectly with an intermediate period [Figure 14]. Type2 and

type3 mutations are also tested, and all the simulations reflect one common phenomenon which is that oscillators with different periods have the ability to sync with an intermediate period [Figure 15, 16, 17, 18]. Currently we do not know the mechanisms that mediate the synchronization, but monomer exchange might be a potential candidate to explain this phenomena.

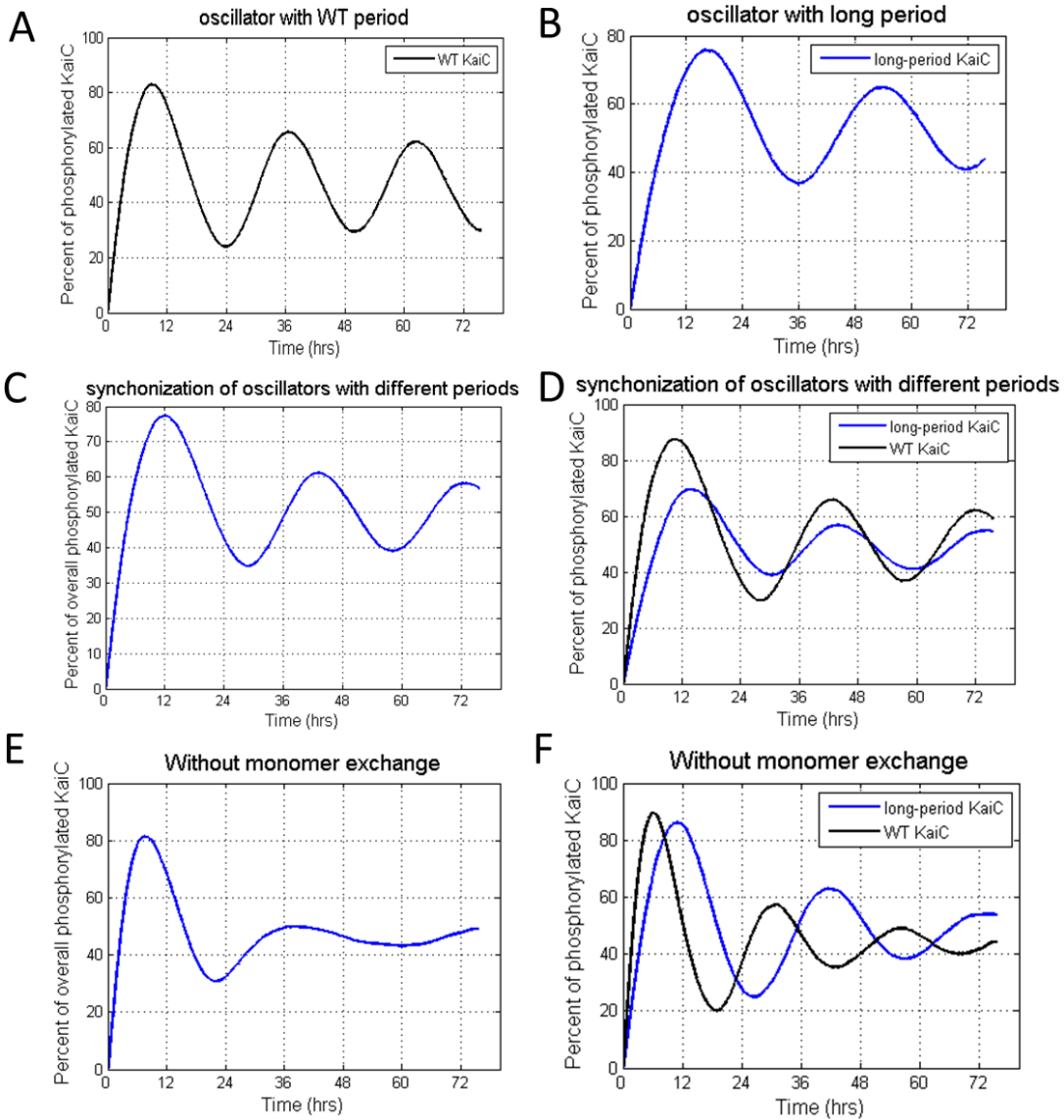


Figure 13. Model prediction of synchronization of oscillators with different periods due to KaiA-KaiC phosphorylation rate decrease as a function of time in the *in vitro* oscillation reaction.

(B) An KaiA-KaiC phosphorylation rate decrease (Type 1) results in a relatively longer period compared to WT, as shown in (A). When monomer exchange is allowed, overall KaiC phosphorylation rhythm (C) or two oscillators separately (D) are simulated after mixing the long-period oscillator and WT oscillator together. (E) When monomer exchange is disallowed, the overall rhythm of KaiC phosphorylation dampens rapidly compared to when ME is allowed (C). (F) long-period and WT oscillators can not synchronize after mixing when ME is disallowed and therefore the rhythm dampens quickly.

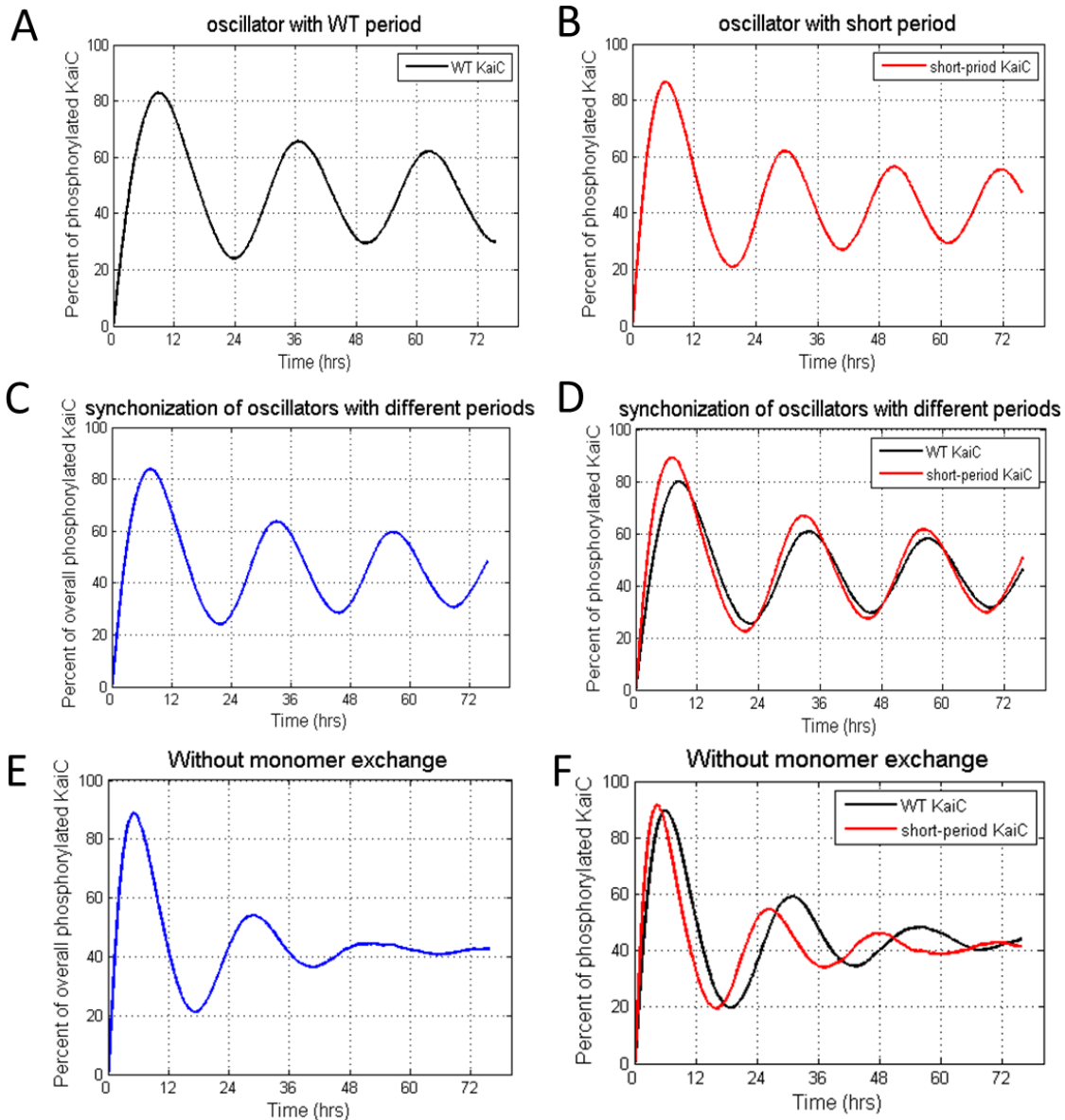


Figure 14. Model prediction of synchronization of oscillators with different periods due to KaiA-KaiC phosphorylation rate increase as a function of time in the *in vitro* oscillation reaction.

(B) An KaiA-KaiC phosphorylation rate increase (Type 1) results in a relatively shorter period compared to WT, as shown in (A). When monomer exchange is allowed, overall KaiC phosphorylation rhythm (C) or two oscillators separately (D) are simulated after mixing the short-period oscillator and WT oscillator together. (E) When monomer exchange is disallowed, the overall rhythm of KaiC phosphorylation dampens rapidly compared to when ME is allowed (C). (F) Short-period and WT oscillators can not synchronize after mixing when ME is disallowed and therefore the rhythm dampens quickly.

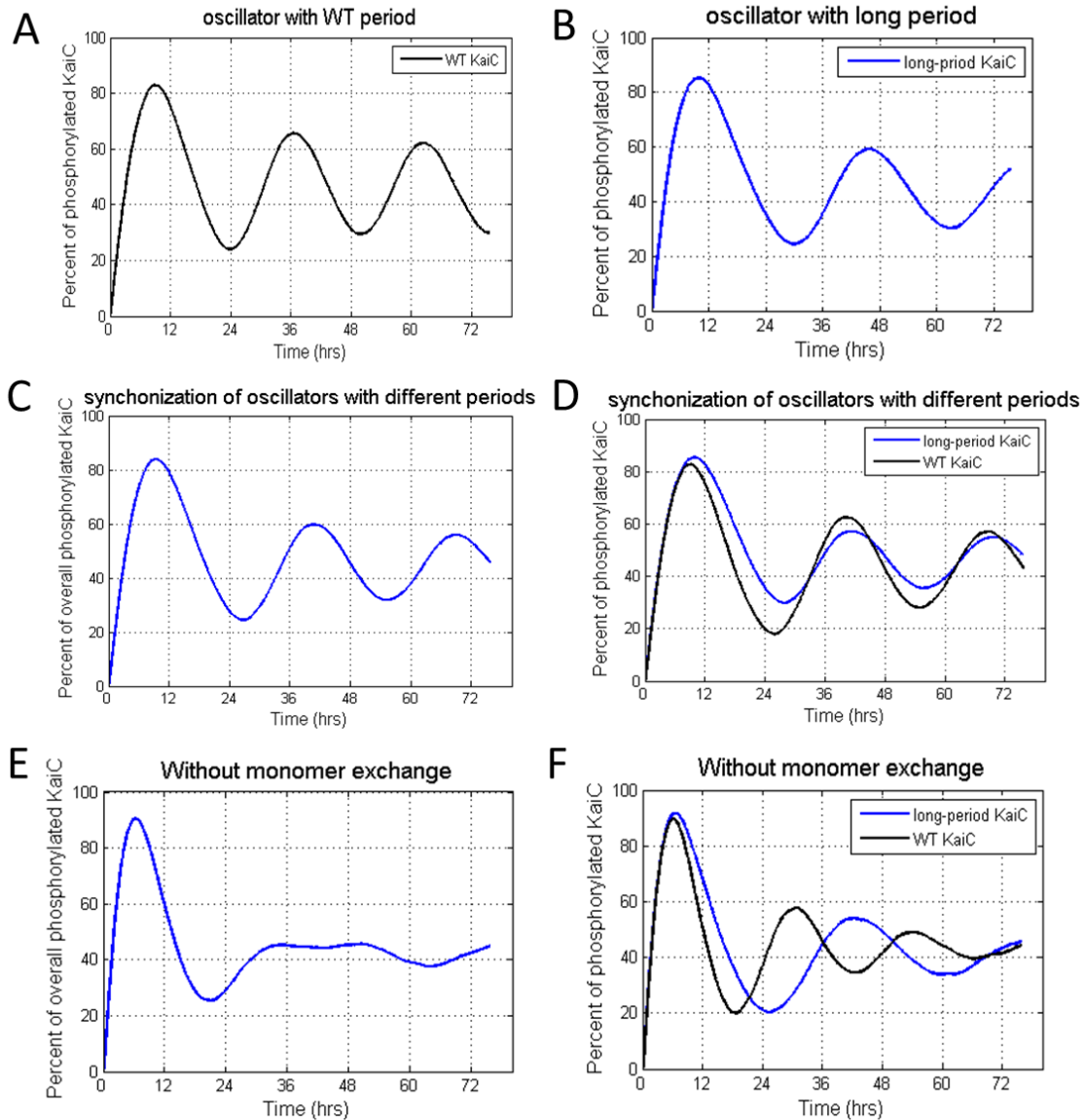


Figure 15. Model prediction of synchronization of oscillators with different periods due to KaiC\* dephosphorylation rate decrease as a function of time in the *in vitro* oscillation reaction.

(B) An KaiC\* dephosphorylation rate decrease (Type 2) results in a relatively longer period compared to WT, as shown in (A). When monomer exchange is allowed, overall KaiC phosphorylation rhythm (C) or two oscillators separately (D) are simulated after mixing the long-period oscillator and WT oscillator together. (E) When monomer exchange is disallowed, the overall rhythm of KaiC phosphorylation dampens rapidly compared to when ME is allowed (C). (F) long-period and WT oscillators can not synchronize after mixing when ME is disallowed and therefore the rhythm dampens quickly.

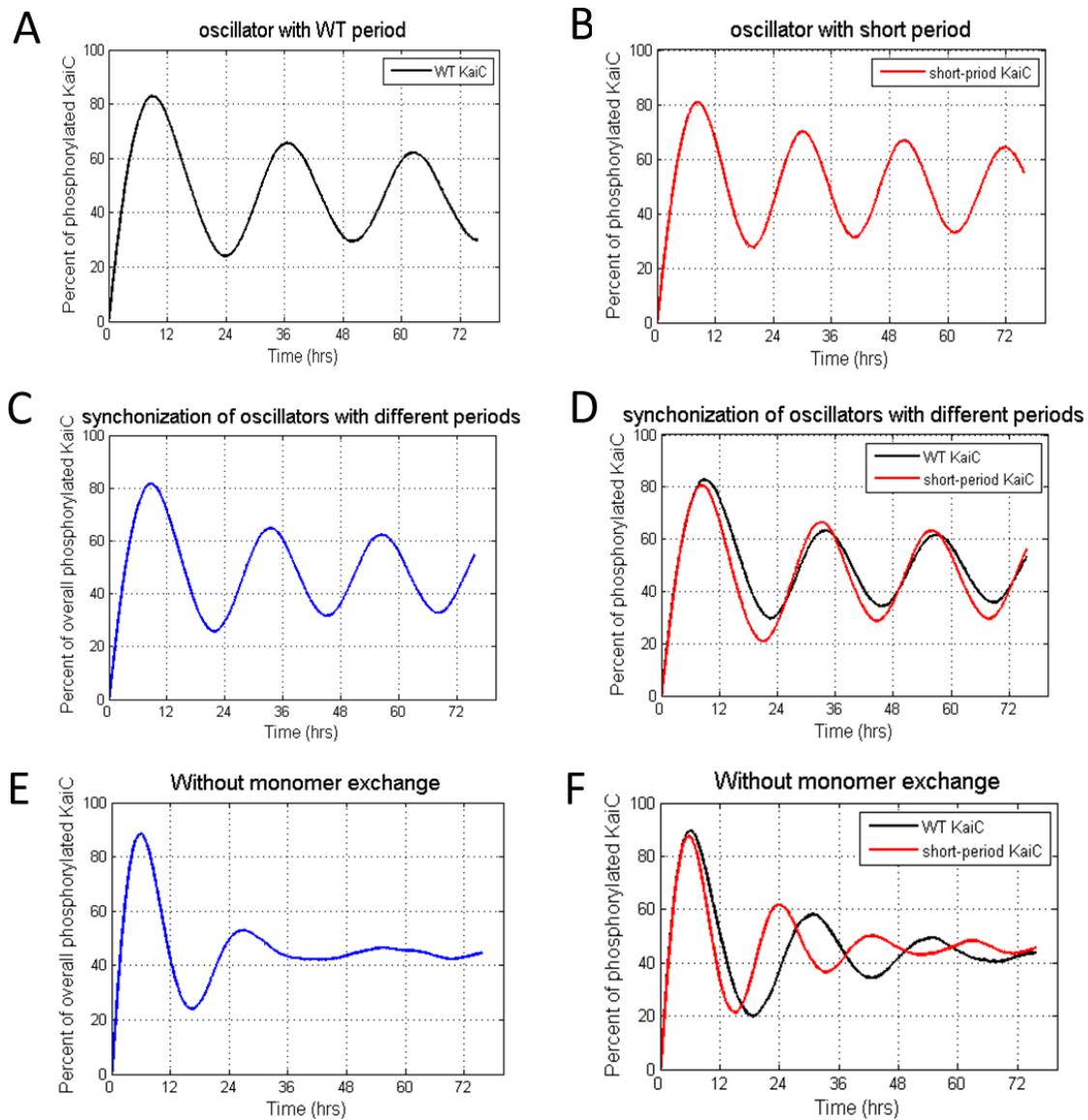


Figure 16. Model prediction of synchronization of oscillators with different periods due to KaiC\* dephosphorylation rate increase as a function of time in the *in vitro* oscillation reaction.

(B) An KaiC\* dephosphorylation rate increase (Type 2) results in a relatively shorter period compared to WT, as shown in (A). When monomer exchange is allowed, overall KaiC phosphorylation rhythm (C) or two oscillators separately (D) are simulated after mixing the short-period oscillator and WT oscillator together. (E) When monomer exchange is disallowed, the overall rhythm of KaiC phosphorylation dampens rapidly compared to when ME is allowed (C). (F) Short-period and WT oscillators can not synchronize after mixing when ME is disallowed and therefore the rhythm dampens quickly.

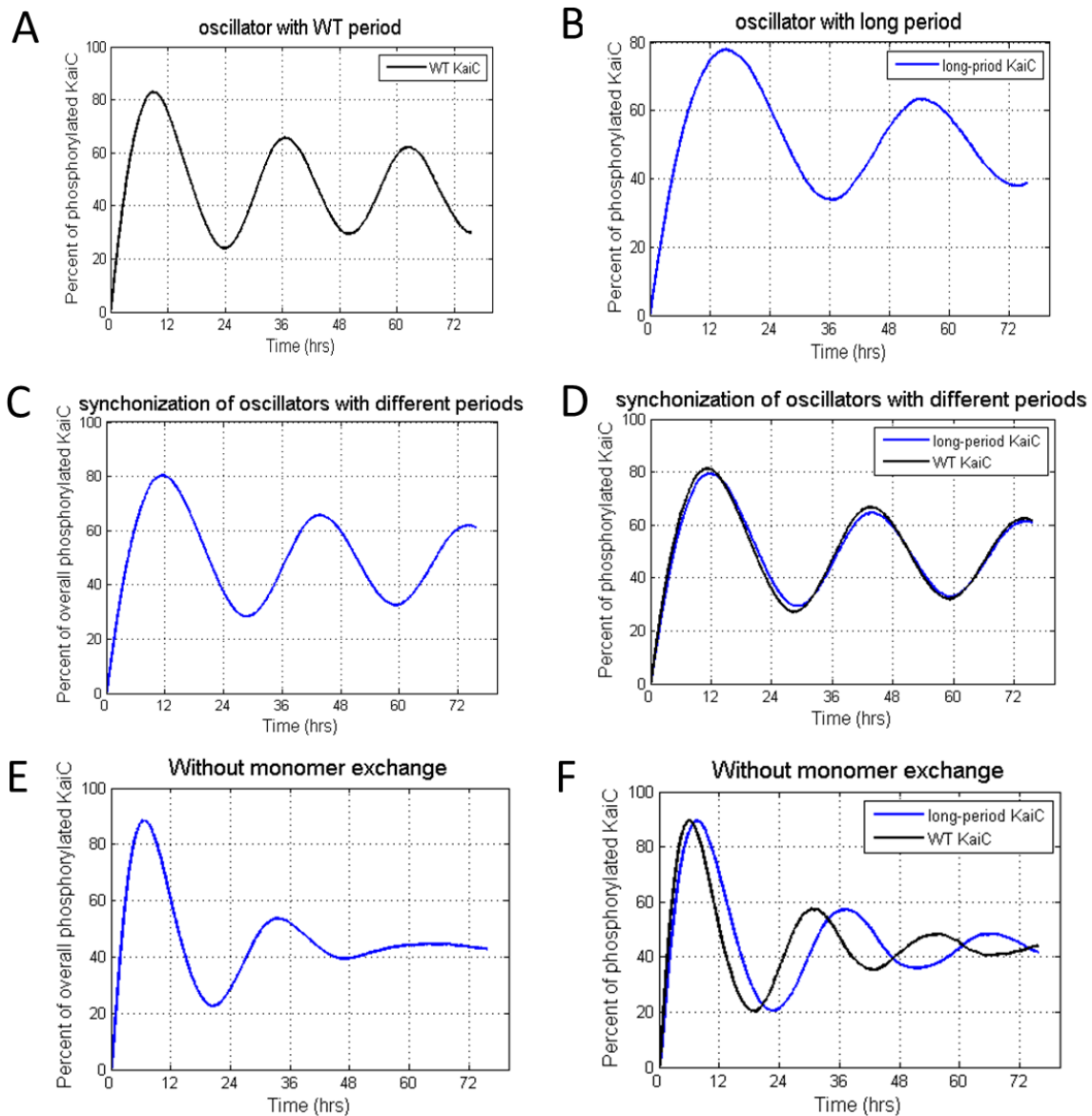


Figure 17. Model prediction of synchronization of oscillators with different periods due to KaiA-KaiC on/off rate decrease as a function of time in the *in vitro* oscillation reaction. (B) An KaiA-KaiC on/off rate decrease (Type 3) results in a relatively longer period compared to WT, as shown in (A). When monomer exchange is allowed, overall KaiC phosphorylation rhythm (C) or two oscillators separately (D) are simulated after mixing the long-period oscillator and WT oscillator together. (E) When monomer exchange is disallowed, the overall rhythm of KaiC phosphorylation dampens rapidly compared to when ME is allowed (C). (F) long-period and WT oscillators can not synchronize after mixing when ME is disallowed and therefore the rhythm dampens quickly.

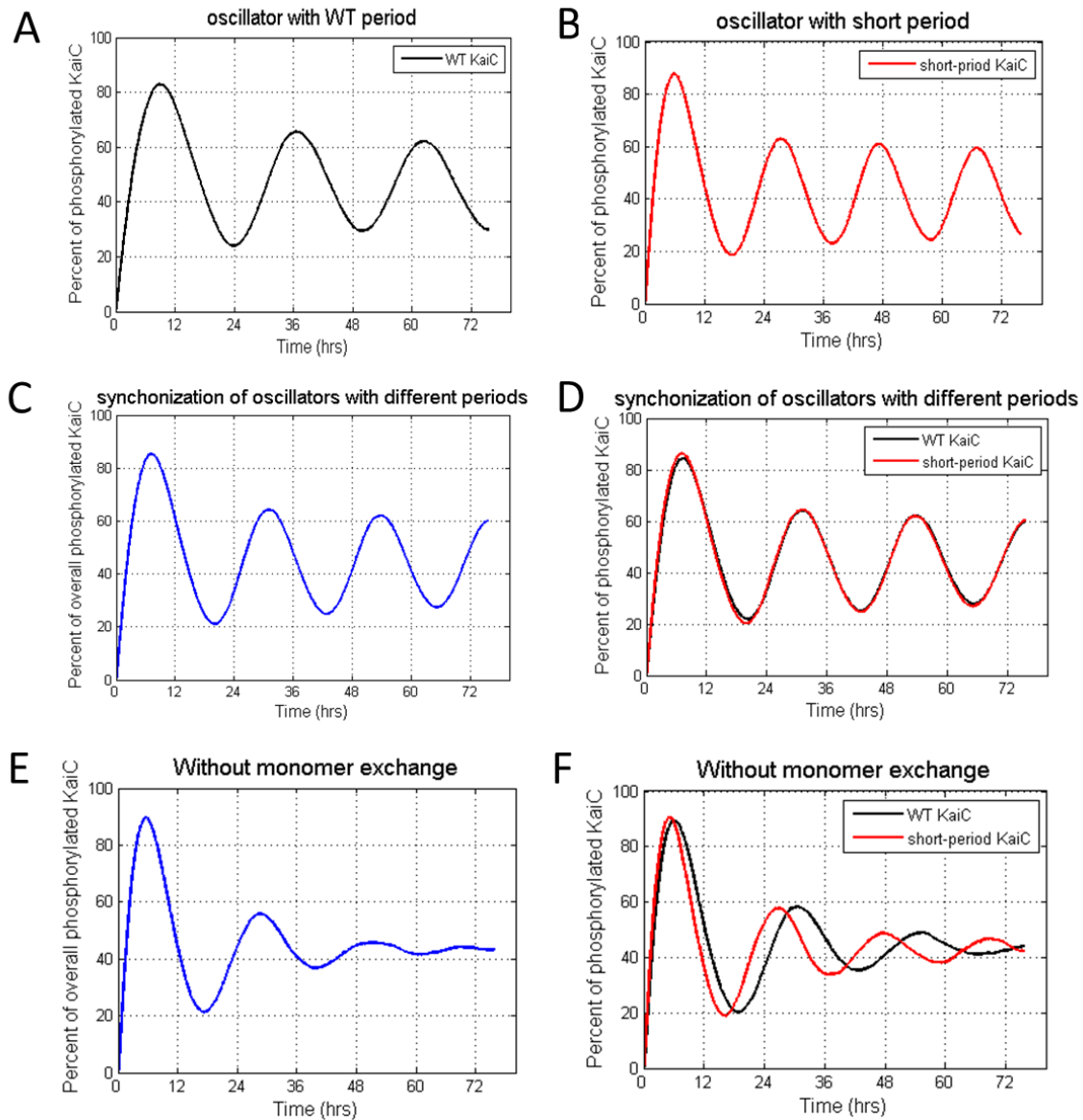


Figure 18. Model prediction of synchronization of oscillators with different periods due to KaiA-KaiC on/off rate increase as a function of time in the *in vitro* oscillation reaction. (B) An KaiA-KaiC on/off rate increase (Type 3) results in a relatively shorter period compared to WT, as shown in (A). When monomer exchange is allowed, overall KaiC phosphorylation rhythm (C) or two oscillators separately (D) are simulated after mixing the short-period oscillator and WT oscillator together. (E) When monomer exchange is disallowed, the overall rhythm of KaiC phosphorylation dampens rapidly compared to when ME is allowed (C). (F) short-period and WT oscillators can not synchronize after mixing when ME is disallowed and therefore the rhythm dampens quickly.



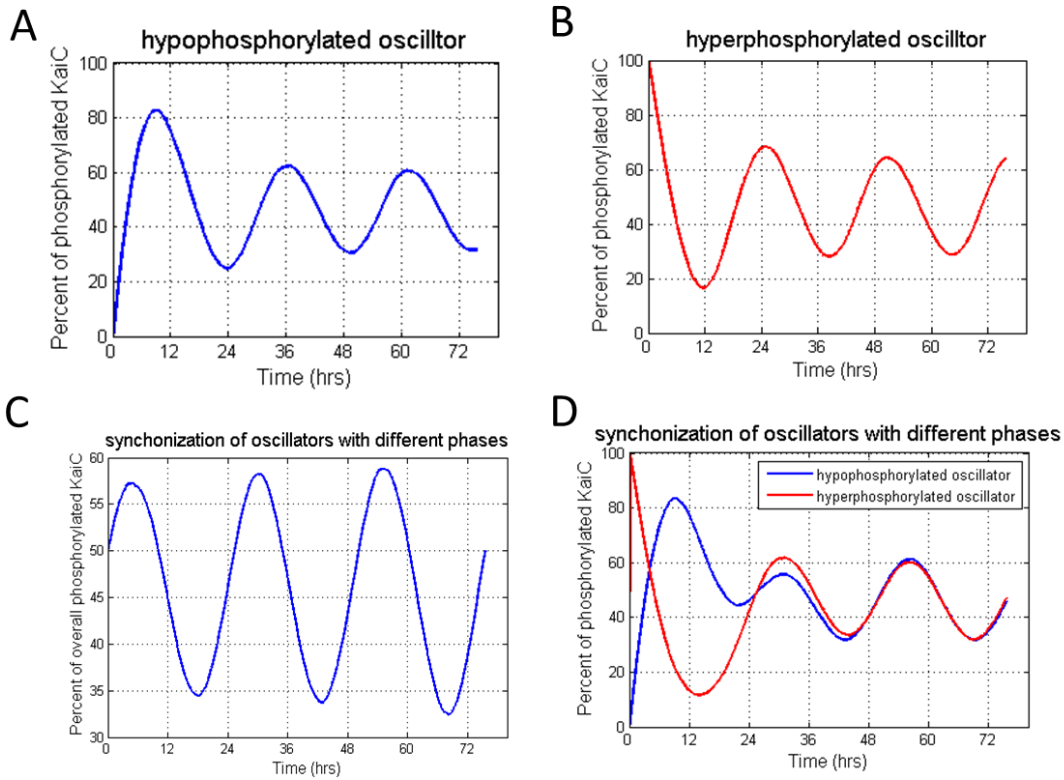


Figure 19. Model prediction of synchronization of oscillators with different phases as a function of time in the *in vitro* oscillation reaction in the presence or absence of monomer exchange.

Two *in vitro* oscillations in opposite phases are simulated. Oscillation originally in hypophosphorylation phase is shown in (A), and the one in hyperphosphorylation phase is shown in (B). When monomer exchange is allowed, overall KaiC phosphorylation rhythm (C) or two oscillators separately (D) are simulated after mixing the two oscillators in opposite phases together.

## Discussion

In this study, we first investigated the synchronization phenomena between two oscillators with different periods *in vivo*. Overall, these data are consistent with the hypothesis that two oscillators with different periods will lead to a robust rhythm with a shared intermediate period, even though the period of the two oscillators are 8-hour apart. Furthermore, we investigated whether increased expression levels of *KaiC* mutant gene will “push” the resulting rhythm to possess a period more like the mutant, in a co-expression *in vivo* experiment. Our hypothesis is that not only two oscillators with different periods can sync with a shared period, but also the resulting period should be determined by the stoichiometry of the two oscillators. This hypothesis is also supported in a way that the resulting rhythm shows a period more like the short-period mutant with the increase of the short-period mutant proteins.

The *in vivo* experiments, however, have raised more questions, since several complicated processes such as transcription and translation are also involved. To investigate the synchronization of central oscillator directly, we have utilized the *in vitro* oscillator. The synchronization between WT oscillator and long-period mutant *in vitro* has echoed the *in vivo* result; however, we encountered several problems in the reconstitution of short-period mutant *in vitro*. For example, the tests of  $KaiC^{T415A}$  with multiple purifications have failed to elicit *in vitro* rhythm. We have used other short-period mutants, for example,  $KaiC^{F470Y}$ , but the problem remained.  $KaiC^{P248A}$  is then included in this assay, which has been confirmed to have a short period *in vitro* (unpublished result by Dr. Mori) [Figure 11]. However, it is a temperature-dependent mutant *in vivo*, with a  $Q_{10}$  of  $\sim 1.3$ , thus making it uncertain how this mutant will behave

*in vitro* as temperature changes. KaiC<sup>WT</sup> and KaiC<sup>P248A</sup> both display rhythms with high amplitude by themselves. However, after mixing, their rhythms retained but dampened severely. It is difficult to determine whether the dampening is due to a synchronization defect in general or to the unique temperature-dependent feature of KaiC<sup>P248A</sup>. In order to optimize this experiment, we need to reconstitute another short-period rhythm *in vitro* which is temperature-compensated.

Computational simulation of the circadian rhythm has been a really helpful method to study this topic. Different models have been proposed to explain the molecular synchronization among KaiC hexamers<sup>32,33,35</sup>. We also tried to employ computational simulation to predict the synchronization phenomena that we have observed *in vivo* and *in vitro*. A matrix model, reported by Mori et al., has been chosen to perform simulation as this model enables us to track two populations of KaiC separately over the entire process.

As expected, the simulated results agreed with the *in vitro* experiment that oscillations with different periods successfully synced together with an intermediate period, while the synchronization could be due to the monomer exchange. However, this matrix model was proposed in 2007 and since then, a number of discoveries on KaiC oscillation have been made while some contradicting with certain assumptions of this model.

1. This model adopts value 3 for the parameter “Maximum number of phosphates per monomer”, whereas more and more researchers tend to agree that every monomer has two phosphorylation sites: T432 and S431<sup>17,18</sup>.

The reported third phosphorylation site T426 is phosphorylatable under some conditions but not stable enough to be detected every time.

2. This model states that the threshold for KaiB binding is 17 phosphates per hexamer, which needs to be reconsidered as well. Phosphorylation of S431 in KaiC plays a key role for KaiB binding, and on the contrary, T432 phosphorylation appears to have no effect on the Kai protein interactions<sup>35,37</sup>. Therefore, the threshold for KaiB binding awaits future experimental evidence.
3. Monomer exchange is permitted between KaiC hexamers in the same conformation, and disallowed between KaiCs in different conformations in this model. However, by using these criteria, when simulating the synchronization of oscillators in different phases, there was a discrepancy between simulation and experimental result [Figure 19D]. The two oscillators rapidly sync their phosphorylation state and reaction direction, in the first 8 hours after mixing, while in the simulation result, two oscillators achieved synchronization over 24 hours. It is plausible that monomer exchange is a prevalent phenomenon among KaiC hexamers. In other words, monomer exchange might occur between all KaiC hexamers, regardless of their conformation. However, it is also likely that other mechanisms including KaiA sequestration are present. KaiA sequestration model proposes that the affinity of KaiA for KaiC tentacles depends on phosphorylation state of KaiC (non-linear KaiA sequestration)<sup>32,33</sup>. Both mechanisms may work together to secure the molecular synchronization of KaiC population in the *in vitro* reaction.

Which defect(s) do our KaiC mutants used in the *in vitro* experiments carry? It is an interesting and important question to ask. Unfortunately, we face a lot of technical difficulties to solve this question. We didn't detect obvious changes of KaiA-KaiC phosphorylation rate between 3myc-KaiC, KaiC<sup>T42S</sup> and KaiC<sup>T415A</sup>, nor KaiC dephosphorylation rate, measured by SDS-PAGE (data not shown). Furthermore, we couldn't find a reliable method to measure KaiA-KaiC on/off rate. Therefore, why these KaiC mutants possess period changes are not known at this point.

## Materials and Methods

### *Strains, Culture Conditions, and Luminescence Assay of In Vivo Rhythms*

The AMC149 strain is wild-type *Synechococcus elongatus* PCC 7942 in which a luciferase gene set reporter (*luxAB*) was introduced downstream of the *psbAI* promoter (*psbAIp::luxAB*) in neutral site I<sup>11</sup>. AMC149 is resistant to spectinomycin, so that we can maintain and select the specific strain with the antibiotics. All strains were grown in BG-11 medium and experiments were performed at 30C except as indicated otherwise.

KaiC gene mutants that were used in the thesis: KaiC<sup>T42S</sup>, KaiC<sup>P248A</sup>, KaiC<sup>T415A</sup>, KaiC<sup>F470Y</sup>, KaiC<sup>T495A</sup>,  $\Delta$ KaiC. KaiC<sup>T42S</sup>, KaiC<sup>P248A</sup>, KaiC<sup>T415A</sup>, KaiC<sup>F470Y</sup>, KaiC<sup>T495A</sup> had a single or double point mutations at different locations in the KaiC gene, which lead to amino acid substitutions from threonine to serine, proline to alanine, threonine to alanine, phenylalanine to tyrosine, threonine to alanine, respectively [Table 1]. KaiC<sup>T42S</sup> was a rhythmic mutant that exhibited a period of luminescence rhythms of approximately 28hr. KaiC<sup>P248A</sup>, KaiC<sup>T415A</sup> and KaiC<sup>F470Y</sup> expressed periods of 20hr, 19hr and 17hr respectively.

The entire *kaiC* gene was substituted with a chloramphenicol antibiotic gene in the  $\Delta$ KaiC strain, and a *luxAB* luminescence reporter under the control of *psbAI* promoter was also introduced to NSII of  $\Delta$ KaiC strain, as in AMC149.

Name	Phenotype (in vivo)	Nucleotide mutation	Amino acid mutation
KaiC <sup>T42S</sup>	28h (41)	124: A>T	42: T>S
KaiC <sup>P248A</sup>	20h(43)	342-744: CCG>GCC	248: P>A
KaiC <sup>T415A</sup>	19h(42)	1243: A>G	415: T>A
KaiC <sup>F470Y</sup>	17h(41)	1409: T>A	470: F>Y
KaiC <sup>T495A</sup>	AR	1483: A>G	495: T>A

Table 1. Phenotypes, nucleotide mutations, and amino acid mutations of different KaiC mutants used in the study.

For assay of in vivo rhythms of *psbAI* promoter activity, luminescence emitted by the *psbAlp::luxAB* from colonies on agar plates was recorded real-time using Kondotron, as previously described<sup>38</sup>. Colonies on agar plates were given 1 light:dark cycles (LD 12:12) to synchronize the cyanobacteria population, and subsequently recorded the free-running luminescence rhythms using JKtron in constant light condition (LL; cool-white fluorescence at 40-50 uE/m<sup>2</sup>s).

#### *Transformation of cyanobacteria*

50ml of cells were grown in BG-11 to log-phase and 10ml of cells were used to do one transformation. Cells were harvested by centrifugation at 3000 rpm for 15 min and suspended and washed in fresh media once. The pellets were suspended again in 0.5ml BG-11 media, and added 1ug of plasmid DNA. Cells were then incubated with mild shaking overnight at 30C covered in aluminum foil. Cells then were plated on a BG-11 plate without any antibiotics, and incubated at 30C under constant illumination

overnight. Proper antibiotics were added to the plates the next day. Colonies appeared were picked and transferred to another fresh plate with proper antibiotics. Every strain was restreaked several times before any experiment.

### *Preparation of Kai proteins*

Kai proteins from *S.elongatus* were expressed in *Escherichia coli* (BL21 cell line) and purified as described by Nishiwaki and coworkers<sup>17</sup> with slight modifications. Site-directed mutagenesis of KaiC to generate KaiC mutants were performed by a modified method of Papworth et al.(1996)<sup>39</sup>. Expression and purification of all KaiC mutants followed the protocol used with wt-KaiC. GST fusion proteins were purified by affinity chromatography on glutathione-agarose resin (Pierce/Thermo Scientific) at 4C. Desired proteins then were cleaved from GST tag using PreScission protease and eluted from the resin. The eluted proteins were further subjected to ion-exchange chromatography on Q sepharose with a gradient of NaCl (from 100mM to 400mM with a 10mM interval). The purity of each Kai protein was assessed by analyzing the sample on SDS-PAGE gels, and protein concentration was measured with the Bradford method (BioRad protein assay dye concentrate) using a dilution series of bovine serum albumin (Bio-Rad to generate a standard curve). KaiC proteins were kept in 20mM Tris-HCl PH8, 150mM NaCl, 5mMMgCl<sub>2</sub>, 1mMATP, 0.5mM EDTA, 1mM DTT. KaiA and KaiB proteins were kept in the same buffer without ATP. All proteins were snap-frozen with liquid nitrogen and stored immediately in -80C freezer.



### *In vitro reactions*

KaiA, KaiB, KaiC proteins were adjusted to a final concentration of 50ng/ul, 50ng/ul and 200ng/ul respectively in the in vitro reactions, with 20mM Tris-HCl PH8, 150mM NaCl, 5mM MgCl<sub>2</sub>, 1mM ATP, 0.5mM EDTA, 1mM DTT. The reaction mixture was dialyzed against the reaction buffer without ATP at 30C for 24 hours to synchronize all the KaiC proteins to de-phosphorylated states. The reactions were initiated the next day by addition of 1 mM ATP and incubated at 30C in a circulating water bath. To assess the phosphorylation state of KaiC, an aliquot of desired volume was taken from the reaction every 4 hours, and mixed with SDS-PAGE loading buffer, and stored at -20C. All samples were boiled at 96C for 10min, and total 1ug KaiC at each time point were subject to 16cm x 16cm x 1mm SDS-PAGE gels (10% acrylamide) at a constant current of 35mA for 4-5 h. Gels were stained with colloidal Coomassie Brilliant Blue. Digital images were analyzed by ImageJ to determine the percentages of hyperphosphorylated and hypophosphorylated KaiC. On each lane of the SDS-PAGE gels, the uppermost band is double phosphorylated KaiC (ST-KaiC), the next band down is KaiC that is phosphorylated on T432 (T-KaiC), the third band is KaiC that is phosphorylated on S 431 (S-KaiC), and the bottommost band is non-phosphorylated KaiC (NP-KaiC).

### *Analysis of protein interactions among three Kai proteins by Native-PAGE*

Native-PAGE gels were used to analyze the interaction between KaiA KaiB and KaiC proteins. For interaction between KaiB and KaiC, KaiB and KaiC proteins were adjusted to 50ng/ul and 200ng/ul final concentration respectively and incubated together

at 30C in water bath. For interaction among all three Kai proteins, KaiA (50ng/ul final concentration) was also added into the reaction. Aliquots (16ul) of the Kai protein mixtures were collected at each desired time point, as indicated in the figures, and combined with 5X native-PAGE sample buffer (50% glycerol, 0.05% bromophenol blue, 0.312M Tris-HCl, PH 6.8), mixed well and flash-frozen in liquid nitrogen, and finally stored at -80C. All samples were allowed to thaw on ice for several minutes on ice before loading. Native-PAGE (10cm x 10cm gels of 7.5% polyacrylamide gels) was performed in cold room at 5mA constant current per gel for 5 h to resolve protein complexes. Running buffer was the same as SDS-PAGE running buffer without SDS, containing only Tris-HCl and Glutamine, and pre-cooled in cold room for at least two hours. Gels were stained with colloidal Coomassie Brilliant Blue, and after de-staining, gel images were digitally captured by Bio-Rad Gel Doc XR system.

### *Model description*

The computational model for synchronization of oscillators used in this thesis was adopted from the matrix model described in 2007<sup>33</sup> with several modifications. In the matrix model, KaiC hexamer kinetics is simulated using an  $N$  by  $(6+3)$  matrix, where  $N$  refers to the number of KaiC hexamers being simulated in the model. The first 6 columns of the matrix refer to the six KaiC monomers in one hexamers and the number of phosphates each monomer carries is recorded as this specific matrix value. The following 3 columns label the association status and conformation of this hexamer, for example, column 7 indicates whether the hexamer is associated with KaiA (0 or 1 value), column 8 indicates whether the hexamer is associated with KaiB (0 or 1 value),

and column 9 labels whether the hexamer is in conformation C or C\* (0 or 1 value). In synchronization simulation, another parallel N by 6 matrix records the origin of the each monomer, which labels whether this specific monomer is originally from population 1 or population 2 in a real-time fashion (0 or 1 value).

Our matrix model adopted following assumptions to describe synchronization phenomenon:

1. The dynamics of KaiC hexamers are simulated using Monte Carlo method, which means that monomers get phosphorylated / dephosphorylated / associated /disassociated with other proteins / monomer exchanged all according to the pre-selected probabilities [Table 2].
2. In the synchronization simulations, monomers are allowed to exchange with others solely depending on the conformational state of both hexamers selected, no matter which population the monomer comes from. In other words, monomers from two different populations (eg. short-period and WT KaiC populations) can monomer exchange as well with selected probability.
3. After monomer from one population get monomer-exchanged to a hexamer belonging to another population, this monomer will behave based on its own traits, for example, its own KaiA on/off rate, phosphorylation rate, dephosphorylation rate and so on, regardless of which population adjacent monomers comes from.

<b>Parameters</b>	
Number of hexamers (each population)	2000
Maximum # of phosphates per monomer	3
Threshold for KaiB binding	17

<b>Reaction</b>	<b>Hexamer State</b>	<b>Probability per second (per molecule)</b>		
		<b>WT</b>	<b>Short-period</b>	<b>Long-period</b>
Phosphorylation	KaiC	7.03E-06	same	same
	KaiA-KaiC	1.69E-04	2.53E-04(type1)	0.84E-04(type1)
	KaiC*, KaiB-KaiC*	1.41E-07	same	same
	KaiA-KaiC*	1.41E-07	same	same
De-phosphorylation	KaiC	2.81E-05	same	same
	KaiA-KaiC	1.41E-06	same	same
	KaiC*, KaiB-KaiC*	7.03E-05	11.25E-05(type2)	4.22E-05(type2)
	KaiA-KaiC*	7.03E-05	11.25E-05(type2)	4.22E-05(type2)
KaiA interactions	KaiA-KaiC, KaiA-KaiC* on	2.25E-03	3.37E-03(type3)	1.12E-03(type3)
	KaiA-KaiC, KaiA-KaiC* off	3.75E-03	5.63E-03(type3)	1.87E-03(type3)
KaiB interactions	KaiB-KaiC, KaiB-KaiC* on	4.69E-04	same	same
	KaiB-KaiC, KaiB-KaiC* off	8.44E-05	same	same
Relaxation	KaiC* -> KaiC	1.88E-04	same	same
Monomer exchange	KaiC-KaiC	3.75E-03	same	same
	KaiC*-KaiC*	4.22E-03	same	same
	KaiC-KaiC*	0.00E+00	same	same

Table 2. Model parameters and rates (modified from Mori et al.<sup>33</sup>)

## REFERENCES

1. Ditty, J.L., S.B. Williams, and S.S. Golden, *A cyanobacterial circadian timing mechanism*. *Annu Rev Genet*, 2003. **37**: p. 513-43.
2. Jay C. Dunlap, J.J.L., Patricia J. DeCoursey, *Chronobiology: Biological Timekeeping*. 2004: Sinauer Associates, Inc.
3. Terman, M., et al., *Light treatment for sleep disorders: consensus report. IV. Sleep phase and duration disturbances*. *J Biol Rhythms*, 1995. **10**(2): p. 135-47.
4. Turek, F.W., et al., *Obesity and metabolic syndrome in circadian Clock mutant mice*. *Science*, 2005. **308**(5724): p. 1043-5.
5. Kondratov, R.V., et al., *Early aging and age-related pathologies in mice deficient in BMAL1, the core component of the circadian clock*. *Genes Dev*, 2006. **20**(14): p. 1868-73.
6. Lucas J. Stal, W.E.K., *Nitrogenase activity in the non-heterocystous cyanobacterium Oscillatoria sp. grown under alternating light-dark cycles*. *Arch Mikrobiol*, 1985(143): p. 67-71.
7. A. MITSUI, S.K., A. TAKAHASHI, H. IKEMOTO, S. CAO & T. ARAI, *Strategy by which nitrogen-fixing unicellular cyanobacteria grow photoautotrophically*. *Nature*, 1986(323): p. 720-722.
8. N. Grobbelaar, T.C.H., H.Y. Lin, T.J. Chow, *Dinitrogen-fixing endogenous rhythm in Synechococcus RF-1*. *FEBS Lett*, 1986. **37**(2): p. 173-177.
9. Johnson, C.H., T. Mori, and Y. Xu, *A cyanobacterial circadian clockwork*. *Curr Biol*, 2008. **18**(17): p. R816-R825.
10. Golden, S.S., et al., *Cyanobacterial Circadian Rhythms*. *Annu Rev Plant Physiol Plant Mol Biol*, 1997. **48**: p. 327-354.
11. T Kondo, C.A.S., R D Kulkarni, W Taylor, M Ishiura, S S Golden, C H Johnson, *Circadian rhythms in prokaryotes: luciferase as a reporter of circadian gene expression in cyanobacteria*. *Proc Natl Acad Sci U S A*, 1993. **90**(12): p. 5672-5676.
12. Kondo, T. and M. Ishiura, *Circadian rhythms of cyanobacteria: monitoring the biological clocks of individual colonies by bioluminescence*. *J Bacteriol*, 1994. **176**(7): p. 1881-5.
13. Kondo, T., et al., *Circadian clock mutants of cyanobacteria*. *Science*, 1994. **266**(5188): p. 1233-6.
14. Ishiura, M., et al., *Expression of a gene cluster kaiABC as a circadian feedback process in cyanobacteria*. *Science*, 1998. **281**(5382): p. 1519-23.

15. Liu, Y., et al., *Circadian orchestration of gene expression in cyanobacteria*. Genes Dev, 1995. **9**(12): p. 1469-78.
16. Iwasaki, H., et al., *KaiA-stimulated KaiC phosphorylation in circadian timing loops in cyanobacteria*. Proc Natl Acad Sci U S A, 2002. **99**(24): p. 15788-93.
17. Nishiwaki, T., et al., *Role of KaiC phosphorylation in the circadian clock system of Synechococcus elongatus PCC 7942*. Proc Natl Acad Sci U S A, 2004. **101**(38): p. 13927-32.
18. Xu, Y., et al., *Identification of key phosphorylation sites in the circadian clock protein KaiC by crystallographic and mutagenetic analyses*. Proc Natl Acad Sci U S A, 2004. **101**(38): p. 13933-8.
19. Mackey, S.R. and S.S. Golden, *Winding up the cyanobacterial circadian clock*. Trends Microbiol, 2007. **15**(9): p. 381-8.
20. Nakajima, M., et al., *Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation in vitro*. Science, 2005. **308**(5720): p. 414-5.
21. Eguchi, K., et al., *Mechanism of robust circadian oscillation of KaiC phosphorylation in vitro*. Biophys J, 2008. **95**(4): p. 1773-84.
22. Qin, X., et al., *Coupling of a core post-translational pacemaker to a slave transcription/translation feedback loop in a circadian system*. PLoS Biol, 2010. **8**(6): p. e1000394.
23. Johnson, C.H., M. Egli, and P.L. Stewart, *Structural insights into a circadian oscillator*. Science, 2008. **322**(5902): p. 697-701.
24. Mori, T., et al., *Circadian clock protein KaiC forms ATP-dependent hexameric rings and binds DNA*. Proc Natl Acad Sci U S A, 2002. **99**(26): p. 17203-8.
25. Iwase, R., et al., *Functionally important substructures of circadian clock protein KaiB in a unique tetramer complex*. J Biol Chem, 2005. **280**(52): p. 43141-9.
26. Johnson, C.H. and M. Egli, *Visualizing a biological clockwork's cogs*. Nat Struct Mol Biol, 2004. **11**(7): p. 584-5.
27. Qin, X., et al., *Intermolecular associations determine the dynamics of the circadian KaiABC oscillator*. Proc Natl Acad Sci U S A, 2010. **107**(33): p. 14805-10.
28. Hardin, P.E., J.C. Hall, and M. Rosbash, *Feedback of the Drosophila period gene product on circadian cycling of its messenger RNA levels*. Nature, 1990. **343**(6258): p. 536-40.
29. Xu, Y., T. Mori, and C.H. Johnson, *Circadian clock-protein expression in cyanobacteria: rhythms and phase setting*. EMBO J, 2000. **19**(13): p. 3349-57.

30. Tomita, J., et al., *No transcription-translation feedback in circadian rhythm of KaiC phosphorylation*. Science, 2005. **307**(5707): p. 251-4.
31. Ito, H., et al., *Autonomous synchronization of the circadian KaiC phosphorylation rhythm*. Nat Struct Mol Biol, 2007. **14**(11): p. 1084-8.
32. van Zon, J.S., et al., *An allosteric model of circadian KaiC phosphorylation*. Proc Natl Acad Sci U S A, 2007. **104**(18): p. 7420-5.
33. Mori, T., et al., *Elucidating the ticking of an in vitro circadian clockwork*. PLoS Biol, 2007. **5**(4): p. e93.
34. Kageyama, H., et al., *Cyanobacterial circadian pacemaker: Kai protein complex dynamics in the KaiC phosphorylation cycle in vitro*. Mol Cell, 2006. **23**(2): p. 161-71.
35. Rust, M.J., et al., *Ordered phosphorylation governs oscillation of a three-protein circadian clock*. Science, 2007. **318**(5851): p. 809-12.
36. Kim, Y.I., et al., *The day/night switch in KaiC, a central oscillator component of the circadian clock of cyanobacteria*. Proc Natl Acad Sci U S A, 2008. **105**(35): p. 12825-30.
37. Nishiwaki, T., et al., *A sequential program of dual phosphorylation of KaiC as a basis for circadian rhythm in cyanobacteria*. EMBO J, 2007. **26**(17): p. 4029-37.
38. Xu, Y., T. Mori, and C.H. Johnson, *Cyanobacterial circadian clockwork: roles of KaiA, KaiB and the kaiBC promoter in regulating KaiC*. EMBO J, 2003. **22**(9): p. 2117-26.
39. Braman, J., C. Papworth, and A. Greener, *Site-directed mutagenesis using double-stranded plasmid DNA templates*. Methods Mol Biol, 1996. **57**: p. 31-44.